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(54) Title: AN INFECTIOUS CLONE FOR HUMAN PARAINFLUENZA VIRUS TYPE 3

(57) Abstract

A system for generating recombinant, human parainfluenza virus, particularly infectious, recombinant, human parainfluenza virus type 3 (HPIV–3) is provided. In one embodiment, the system comprises a clone comprising a nucleotide sequence that encodes a full–length, positive sense, anti–genome of HPIV, and at least one support clone comprising a nucleotide sequence that encodes the HPIV P protein and the HPIV L protein. In another embodiment, the system further comprises a support clone which comprises a nucleotide sequence that encodes the HPIV NP protein. The present invention also provides a clone which comprises a nucleotide sequence encoding the full–length, positive sense, anti–genome of HPIV–3. The clone also comprises an RNA polymerase promoter operatively linked to the HPIV–3 antigenome–encoding sequence. In a preferred embodiement, the clone further comprises a nucleotide sequence which encodes a ribozyme immediately downstream from the sequence encoding the HPIV–3 anti–genome. The present invention also relates to a method of preparing recombinant HPIV–3 virus having site–specific mutations in the HPIV–3 genome. The method comprises preparing a clone comprising a modified HPIV–3 antigenome–encoding sequence; introducing the modified HPIV–3 clone and support clones which comprise nucleotide sequences encoding an HPIV–3 P protein, an HPIV–3 L protein, and, preferably, an HPIV–3 NP protein into host cells; and culturing the host cells under conditions that allow for synthesis of the modified HPIV–3 antigenome and the L, P, and NP proteins of HPIV–3.

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An Infectious Clone for Human Parainfluenza Virus Type 3

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BACKGROUND

Recognized in 1956 as a cause of respiratory infection in man, human parainfluenza viruses (HPIV) are believed to account for 4 to 22 percent of the respiratory illnesses in children, second only to respiratory syncytial virus in this regard. HPIV are important causes of the lower respiratory tract diseases such as pneumonia and bronchiolitis, and are the most common cause of croup in young children. Of the four HPIV serotypes, 1-4, type 3 virus (HPIV-3), appears to be the most virulent, frequently causing bronchiolitis and pneumonia during the first month of life.

Unfortunately, effective vaccines or antiviral therapies, which can be used to prevent or treat HPIV-induced infections, are not presently available. Standard methods which are used to produce inactive viruses, such as heat inactivation or chemical treatment of the virus, have been unsuccessful with all HPIV strains and serotypes, including HPIV-3. Moreover, standard methods for producing attenuated viruses produce mutations at random sites and do not allow one to modify the HPIV genome at specific sites or to control the number of mutations that are introduced into genome.

Human parainfluenza viruses are enveloped, single-stranded, negative sense RNA viruses that are members of the paramyxovirus genus within the family Paramyxoviridae. Replication of the human parainfluenza viral genome (vRNA) is similar to that of other members of the Paramyxoviradae family. Upon infection of a cell, transcription is the major RNA synthetic event, resulting in the production of the viral mRNAs from the negative-sense genome, i.e., the vRNA. Later in infection a transition to RNA replication occurs, resulting in synthesis of a full-length, antigenomic, positive-sense RNA, which serves as the template for synthesis of additional negative-sense genomic RNA. Transcription and replication of the genomic RNA is dependent upon formation of a ribonucleoprotein complex (RNP) consisting of the 15462 nucleotide genomic RNA encapsidated by the nucleocapsid protein (NP), and the closely associated phosphoprotein (P), and the large (L) polymerase protein. Several host cell factors are also involved in the replicative cycle of HPIV.

The requirement for an intact RNP for HPIV has hindered analysis of HPIV transcription and replication in a cell-free system. Efforts to encapsidate HPIV-3 vRNA in vitro have failed, and unlike the positive sense RNA viruses, naked HPIV vRNA is not infectious. Moreover, there currently are no known systems for preparing recombinant HPIV, including recombinant infectious HPIV-3.

Accordingly, there is a need for new reagents, systems, and methods that enable one to produce a recombinant HPIV, particularly a recombinant, infectious HPIV-3. Recombinant systems that permit one to introduce one or more site-specific mutations into the genome of HPIV, particularly HPIV-3, are desirable. Recombinant systems which allow one to characterize the effect of site-specific mutations on the transcription or replication of human parainfluenza viral RNA and to identify the site specific mutations which lead to the production of attenuated HPIV are especially desirable.

SUMMARY OF THE INVENTION.

In accordance with the present invention a system for generating recombinant, human parainfluenza virus, particularly infectious, recombinant, human parainfluenza virus type 3 (HPIV-3) is provided. In one embodiment, the system comprises a clone comprising a nucleotide sequence that encodes a full-length, positive sense, anti-genome of HPIV, and at least one support clone comprising a nucleotide sequence that encodes the HPIV P protein and the HPIV L protein. In another embodiment, the system further comprises a support clone which comprises a nucleotide sequence that encodes the HPIV NP protein. Preferably, each of the clones in the system comprises an RNA polymerase promoter which is operatively linked to the respective HPIV nucleotide sequence contained within the clone.

The present invention also provides a clone which comprises a nucleotide sequence encoding the full-length, positive sense, anti-genome of HPIV-3. The clone also comprises an RNA polymerase promoter operatively linked to the HPIV-3 antigenome-encoding sequence. In a preferred embodiment, the clone further comprises a nucleotide sequence which encodes a ribozyme immediately downstream from the sequence encoding the HPIV-3 anti-genome.

The present invention also relates to a method of preparing recombinant HPIV-3 virus having site-specific mutations in the HPIV-3 genome. The method comprises preparing a clone comprising a modified HPIV-3 antigenome-encoding sequence; introducing the modified HPIV-3 clone and support clones which comprise nucleotide sequences encoding an HPIV-3 P protein, an HPIV-3 L protein, and, preferably, an HPIV-3 NP protein into host cells.; and culturing the host cells under conditions that allow for synthesis of the modified HPIV-3 antigenome and the L, P, and NP proteins of HPIV-3.

The ability to produce recombinant, HPIV-3 virus genetically engineered to contain site-specific mutations within the HPIV-3 genes and cis-acting elements expedites the study of all aspects of the virus replication cycle. Additionally, a system which permits production of recombinant HPIV that is genetically engineered to contain site-specific mutations within the HPIV-3 genome is useful for identifying attenuating parainfluenza genotypes and for developing a live vaccine for human parainfluenza virus.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 depicts the DNA form of the nucleotide sequence of the HPIV-3 genome and shows the location of restriction sites, the leader sequence, the trailer sequence, and the protein encoding regions of the genome.

Figure 2 is a restriction map of the pOCUS-2™ vector.

Figure 3 is a restriction map of pMG(+) showing the location of the leader sequence, luciferase encoding region, and theT7 promoter and terminator.

Figure 4 is a restriction map of pHPIV-3 showing the location of the leader sequence and the protein encoding regions of HPIV-3.

Figure 5 is a schematic depiction of the full-length infectious clone, pHPIV-3. VVϕ, vaccinia virus polymerase stop signal (TTTTTNT); T7, T7 RNA polymerase promoter; le, HPIV-3 leader sequence; NP, P, M, F, HN and L are the HPIV-3 protein coding regions; tr, HPIV-3 trailer sequence; Rz, the hepatitis delta virus antigenomic ribozyme; T7ϕ, T7 RNA polymerase terminator signal. Regions containing substitution mutations are expanded and shown above with the specific changes indicated. The A to G change at viral base 94 creates a *Sac*I site and the A to G change at viral base 15389 creates a *Stu*I site.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention a system for generating recombinant, human parainfluenza virus is provided. In a preferred embodiment, the system is used to generate recombinant HPIV-3. The system comprises a clone comprising a nucleotide sequence, preferably a double-stranded DNA sequence, which encodes a full-length, positive sense anti-genome of HPIV hereinafter referred to as the "HPIV clone", and one or more support clones which comprise nucleotide sequences that encode an HPIV P protein and an HPIV L protein. The nucleotide sequences that encode the HPIV P protein and HPIV L protein may be within the same clone. However, for ease of manipulation, it is preferred that the nucleotide sequences that encode the HPIV P protein and the HPIV L protein be on separate clones. Preferably the HPIV clone comprises a sequence encoding an HPIV-3 antigenome. Preferably the support clone or clones encode a P protein and an L protein of HPIV-3. In another embodiment the system further comprises a support clone which comprises a nucleotide sequence that encodes the HPIV NP protein, preferably the HPIV-3 NP protein.

As used herein "clone" refers to double-stranded DNA that can be introduced into a cell and expressed. The clone may be in the form of a viral vector such as, for example, a vaccinia viral vector, or, preferably, in the form of a plasmid. Preferably, the HPIV clone and the support clones each comprise an RNA polymerase promoter, more preferably a T7 RNA polymerase promoter. Each of the RNA polymerase promoters is operatively linked to the corresponding HPIV encoding sequence in the clone. Thus, the RNA polymerase promoter on the HPIV clone is operatively linked to the HPIV sequence and the RNA polymerase on the support clones are operatively linked to the sequence or sequences encoding

the respective HPIV protein. Preferably, the plasmids comprising the clone also comprise an origin of replication, particularly a bacterial origin of replication.

The present invention also provides a clone which comprises a nucleotide sequence encoding the anti-genomic sequence of HPIV-3, hereinafter referred to as the "HPIV-3 clone". Preferably, the HPIV-3 clone encodes a full-length antigenomic sequence of HPIV-3. As used herein "full-length" means that the anti-genomic sequence is complementary to the entire negative sense, genomic sequence of HPIV-3 extending from the 3' nucleotide of the leader sequence through the 5'nucleotide of the trailer sequence of the HPIV-3 genome. The DNA form of the full-length, genomic sequence of HPIV-3, SEQ ID NO:1, is shown in Fig. 1. In addition to the leader and trailer sequences, the HPIV-3 clone contains sequences encoding the HPIV-3 proteins N, P, M, F, HN, and L, as well as the cis-acting elements. The HPIV-3 clone may encode a wild-type HPIV-3 antigenome sequence or a modified HPIV-3 antigenome having one or more mutations contained therein. The mutation may be in the form of a foreign gene which is inserted into the HPIV-3 antigenome-encoding sequence. Preferably, the mutations are substitutions of one or more nucleotides, deletions of 6 to 12 nucleotides, or additions of 6 to 12 nucleotides in the HPIV-3 antigenome-encoding sequence. More preferably, the modified HPIV-3 clone contains substitutions either in the genes or the cis-acting elements, or both of the HPIV-3 antigenome-encoding sequence.

Preferably, the HPIV-3 clone is a plasmid that comprises a nucleotide sequence which encodes a ribozyme, more preferably the hepatitis delta virus antigenomic ribozyme, immediately downstream from the HPIV antigenome-encoding sequence. Following transcription of the clone, the ribozyme cleaves the ribozyme from the HPIV antigenome to provide a replication competent 3' end on the antigenome. More preferably, the HPIV-3 clone also comprises an RNA polymerase terminator following the ribozyme sequence. In one embodiment, the HPIV-3 clone is the plasmid pHPIV-3 depicted in Fig. 5, which plasmid was deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, USA, on May ___, 1998 and has Accession Number ____.

The present invention also relates to a method of preparing recombinant HPIV, particularly HPIV-3, using the above described system. The method comprises introducing an HPIV clone and the support clones which encode HPIV P protein and HPIV L protein, into host cells, preferably human cells; culturing the host cells under conditions that allow for formation of an HPIV anti-genomic transcript, synthesis of the HPIV genome (vRNA) and the HPIV proteins L, P, and NP, and formation of a recombinant HPIV; and recovering the recombinant HPIV from the culture. Preferably the host cells which are transfected with the HPIV clone and support clones, contain an RNA polymerase that corresponds to the RNA polymerase promoter that is operatively linked to the HPIV sequences in the HPIV clone and the support clones. In a preferred embodiment, a support clone comprising the nucleotide sequence which encodes the HPIV-NP protein operatively linked to an RNA polymerase promoter is also introduced into the cells. Preferably, the host cells are infected with a viral recombinant, preferably a vaccinia virus recombinant, which expresses the RNA polymerase, more preferably the T7 RNA polymerase, prior to or

in combination with transfection with the HPIV clone and support clone or clones. When such cells are infected with the vaccinia virus recombinant, it is preferred that the HPIV-3 clone also comprise a vaccinia virus RNA polymerase terminator upstream of the T7 RNA polymerase and a vaccinia virus RNA polymerase terminator downstream of the T7 RNA polymerase terminator.

The present invention also relates to a method of introducing site-specific mutations into the genome of a recombinant HPIV-3. The method comprises preparing a a modified HPIV-3 clone comprising one or more mutations in the sequence which encodes the HPIV-3 anti-genome; introducing the modified HPIV-3 clone and support clones comprising sequences which encode HPIV-3 P protein and HPIV-3 L protein, and preferably, HPIV-3 NP protein into host cells; and culturing the host cells under conditions that allow for formation of a modified HPIV-3 antigenomic transcript and synthesis of the HPIV-3 L, P, and NP proteins. The modified HPIV-3 clone and the support clones contain an RNA polymerase promoter that is operatively linked to the HPIV-3 protein-encoding sequences. The host cells contain within the cytoplasm thereof an RNA polymerase that corresponds to the RNA polymerase promoter on the modified HPIV-3 clone and the support clones.

Preferably, the modified HPIV-3 clone, containing one or more mutations therein, is made by conventional PCR techniques using an HPIV-3 clone as a template. The mutations are made in the cis-acting elements of the HPIV-3 sequence or in an HPIV-3 protein encoding sequence. Preferably the mutation is made in the L protein-encoding sequence. If mutations are made in the HPIV-3 protein-encoding sequences of the HPIV-3 clone, it is preferred that a similar type of mutation be made in the same site in the protein encoding sequence of the corresponding support clone. For example, if a mutation is made at a specific site in the L protein-encoding sequence of the HPIV-3 clone, it is preferred that the same mutation be made at the same site in the L protein-encoding sequence of the L protein-encoding support clone. Such method is useful for identifying mutations that block the synthesis of viral particles or result in the production of non-infectious or non-virulent HPIV-3.

To determine whether the mutated viruses produced by the above-described method are non-virulent, i.e., attenuated, the mutated viruses are first tested in vitro to determine whether the mutation has resulted in a slower growing phenotype, i.e., the mutated virus grows more slowly in tissue culture than the wild-type virus. The mutated viruses which exhibit this phenotype are then examined in vivo, by injection into an animal, such as the cotton rat, which is good experimental model for parainfluenza virus. The infected animals are then examined to determine if they are producing antibodies to HPIV-3 and to determine if there is a reduction in the severity of symptoms as compared to animals infected with wild-type virus.

The ability to produce recombinant HPIV-3 virus genetically engineered to contain specific alterations within the HPIV-3 genes and cis-acting elements expedites the study of all aspects of the virus replication cycle. Additionally, a system which permits production of recombinant HPIV that is

genetically engineered to contain specific alterations within the HPIV-3 genes is useful for identifying attenuating parainfluenza genotypes and for developing a live vaccine for human parainfluenza virus.

The following examples of methods of preparing a full-length cDNA clone of HPIV-3 and methods of preparing a modified or mutated, infectious, recombinant HPIV -3 are for purposes of illustration and are not intended to limit the scope of the invention.

Example 1. Construction of a Full-Length cDNA Clone of HPIV-3.

The construction of a full-length infectious clone of HPIV-3 containing mutations at specific sites was achieved by a two-step process. The initial step was the generation of a minireplicon which contained the positive sense leader portion region and trailer regions of HPIV-3. The second step involved the insertion of RT-PCR fragments derived from HPIV-3 genomic RNA into the minireplicon.The positive-sense minireplicon contained the following: A T7 promoter which directed the synthesis of two non-viral G residues, followed by the positive-sense leader region of HPIV-3, a portion of the NP 5' UTR (to viral base 97), the luciferase gene, a portion of the L 3'UTR (starting at viral base 15387) and trailer sequences of HPIV-3. The full-length nucleotide sequence of HPIV-3 genome and the location of the leader sequence, trailer sequence and protein-encoding regions is shown in Figure 1. The hepatitis delta virus antigenomic ribozyme followed to effect precise cleavage after the 3 terminal HPIV-3 specific base. A T7 RNA polymerase terminator was also incorporated into the replicon followed the ribozyme sequence. Additionally, vaccinia virus polymerase termination signals were inserted immediately upstream and downstream of the aforementioned sequences. During the construction, single base changes were created in the regions encoding the NP 5' UTR and the L 3 UTR. An A to G change at viral base 94 and the A to G change at base 15389 created SacI and StuI sites, respectively, which served as genetic tags to identify virus as being of recombinant origin.

The vector pOCUS-2 (Novagen) was chosen as the starting plasmid for preparing the mini-replicon [pPIV3-MG(+)], because of its small size (1930 bp). It is believed that the use of a small starting plasmid may increase the stability of the full-length clone.

The mini-replicon was constructed by generating PCR products encoding the leader and trailer regions flanked by a T7 promoter and hepatitis delta virus antigenomic ribozyme, respectively. The primers used for synthesis of the T7 promoter/leader region were: 5'-TAGTCGGCCCTAATACGACTCACTATAGGACCAAACAAGAGAAGAA ACT-3', SEQ ID NO:2, and 5'-GAAATTATAGAGCTCCCTTTTCT-3', SEQ ID NO:3. The first primer encodes an EagI site and the T7 promoter (underlined) and the second primer introduced an A to G base change at viral base 94, (bold) within the 5' untranslated region (UTR) of the NP mRNA, which creates a SacI site. The template for this reaction pHPIV3-CAT, described in De, B. P. and A. K. Banerjee. (1993.) Rescue of synthetic analogs of genome RNA of human parainfluenza virus type 3. Vir. 196:344-348, which is incorporated herein by reference. The resulting PCR product was cloned into the EagI and SacI

sites of pOCUS-2, which is depicted in Figure 2. The primers used for synthesis of the trailer/ribozyme region were:

5'-TAAGGCCTAAAGATAGACAAAAAGTAAGAAAAACATGTAATATATATA

TACCAAACAGAGTTCTTCTCTTGTTTGGTGGGTCGGCATGGCATCTC-3',

SEQ ID NO:4, and 5'-CTGGGTACCTCCCTTAGCCATCCGAGT-3', SEQ ID NO:5. The first primer contains sequence from the 3' UTR of the L mRNA, through the trailer, and primes synthesis of the ribozyme (underlined). Also, an A to G change at viral base 15389 (bold), which creates a StuI site within the 3'UTR of the L mRNA is encoded by this primer. The second primer encodes the 3' end of the ribozyme (underlined) and a BglII site. The template for this PCR reaction was pSA1, a plasmid containing the ribozyme sequence, as previously described in Perrotta, A. T. and M. D. Been. 1991. The pseudoknot-like structure required for efficient self-cleavage of hepatitis delta virus RNA. Nature 350:434-436, which is incorporated herein by reference. The PCR product derived from this reaction was cloned into the StuI and BglII sites of pOCUS-2. The leader and trailer regions were combined into a single clone by transferring the EagI/PstI fragment of the T7/leader clone into the PacI/PstI sites of the trailer/ribozyme clone.

To prevent possible interference by transcription from cryptic vaccinia virus promoters, vaccinia virus polymerase transcription stop signals (TTTTTNT) were inserted upstream and downstream of the replicon near PvuII and SspI sites within pOCUS-2. A T7 transcription termination signal was removed from pET-17b by digesting with BlpI and BspEI, and inserted into the SspI site (blunted with T4 DNA polymerase) of pOCUS-2. A luciferase reporter gene was then inserted into the SacI and StuI sites to create pPIV3-MG(+), which is schematically depicted in Figure 3.

To generate the full-length HPIV-3 clone, five RT-PCR products were generated from HPIV-3 virion RNA and cloned. These fragments were subsequently inserted into pPIV3-MG(+), replacing the luciferase coding sequences, to create pHPIV-3, the full-length clone. The five RT-PCR products were generated from HPIV-3 strain 47885 virion RNA which was obtained from Robert Chanock, at the National Institues of Health. These PCR products, encompassing the remainder of the HPIV-3 genome, were identified by restriction enzyme analysis and cloned, either in pUC19 or pOCUS-2, and then inserted into pPIV3-MG(+).

The first PCR product containing viral bases 83 to 2721 was inserted into the SmaI site of pUC19. The 83/2721 clone was then digested with SacI and XmnI, removing viral bases 94 to 553 which were inserted into the SacI and SphI (blunt with T4 DNA polymerase) of pPIV3-MG(+). The 83/2721 clone was then digested with PstI to remove a fragment containing viral bases 540 to 2274, which was then inserted into the PstI site of the pPIV3-MG(+) clone containing the 94/554 fragment. The second PCR product encompassing viral bases 13395 to 15397 was cloned into the SmaI site of pUC19. This 13395/15397 clone was then digested with StuI and PacI and the resulting fragment containing viral bases

13632 to 15381 was inserted into the StuI and PacI sites of the pPIV3-MG(+) clone containing viral sequence to base 2274. The resulting clone contained viral bases 1 to 2274 and 13632 to 15462 in the pPIV3-MG(+) context.

The third PCR product containing viral bases 7403 to 11513 was digested with BspMI (blunted with T4 DNA polymerase) and XhoI to produce a fragment containing bases 7437 to 11444 which was inserted into the XhoI and SspI sites of pOCUS-2. The fourth PCR fragment containing viral bases 10904 to 13773 was digested with PvuII and BamHI (viral bases 10918 to 13733) and inserted into the EcoRI (blunted with T4 DNA polymerase) and BamHI sites of pUC19. The two viral segments were combined by digesting the 7437/11444 clone with SacI (blunted with T4 DNA polymerase) and EcoNI and inserting into the 10918/13733 clone digested with EcoRI (blunted with T4 DNA polymerase) and EcoNI. The resulting clone contained viral bases 7437 to 13733 in a pUC19 background. The remainder of the viral sequence was derived from a fifth PCR product encompassing viral bases 83 to 7457 which had been digested with XmnI and XhoI (viral bases 553 to 7437) and cloned into the StuI and XhoI sites of pOCUS-2. The 7437/13733 clone was then digested with BamHI, blunted with T4 DNA polymerase, and digested with XhoI to release a fragment that was inserted into the EagI (blunted with T4 DNA polymerase) and XhoI digested 553/7437 clone. The resulting clone contained viral bases 553 to 13733. This clone was then digested with PshAI and PacI and the resulting fragment containing viral bases 2143 to 13632 was inserted into the same sites of the pPIV3-MG(+) clone containing viral bases 1 to 2274 and 13632 to 15462. This generated pHPIV-3, the infectious clone, which is schematically depicted in Figure 4.

To insert the P gene into pGEM-4, P sequences were transferred from a P-lac-fusion clone, (described in 38, which is incorporated herein by reference) by digesting with XbaI (blunted with T4 DNA polymerase) BamHI, and inserted into the KpnI (blunted with T4 DNA polymerase) and BamHI sites of pGEM4. The pPIV3-NP and pPIV3-L clones, as described in 15, 16, which are incorporated herein by reference., were in a pGEM-4 background. The pPIV3-L clone was also modified. In the natural L mRNA sequence there is a non-initiating AUG 11 nucleotides from the 5' end of the transcript. This was removed from pPIV3-L by mutational PCR, changing viral bases 8636 and 8637 from AT to TA.

Example 2. Preparation of Recombinant HPIV-3.

Confluent monolayers of HeLa cells in 6-well plates were infected with recombinant vaccinia virus vTF7-3 at a multiplicity of infection of 2. vTF7-3 expresses T7 RNA polymerase as described in. Fuerst, T. R., P. L. Earl, and B. Moss. 1987. "Use of a hybrid vaccinia virus-T7 RNA polymerase system for expression of target genes." Mol. Cell. Biol. 7:2538-2544, and Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss. 1986. "Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase." Proc. Natl. Acad. Sci. 83:8122-8126, which are incorporated herein by reference. After 1 hour at 37°C, pPIV3-NP, pPIV3-P, pPIV3-L and pHPIV-3 were transfected using Lipofectin (BRL) according to manufacturers instructions. After three hours the

transfection medium was removed and replaced with 1.5 ml Dulbecco's modified Eagle's medium (DMEM)/5% fetal bovine serum. After 40 to 48 hours the plates were frozen, thawed and scraped. The clarified medium supernatant (250 µl) was then used to infect fresh HeLa cell monolayers in 6-well plates. DMEM (1.5ml) containing 25 µg/ml 1-B-D-arabinofuranosylcytosine (araC) to inhibit vaccinia virus replication was added after a 1 hour attachment. After forty hours the plates were frozen, thawed, and scraped. The clarified medium supernatant was then titered for HPIV-3 in the presence of AraC. During the titering, isolated plaques were picked as agar plugs. The agar plugs were placed in 500 ml opti-MEM at 4°C for 4 hr. 250 µl were then used to infect fresh HeLa cell monolayers for amplification of the plaque isolates for 40 hr.

In a preferred embodiment transfection conditions were 1 µg pHPIV-3, 2 µg pPIV3-NP, 4 µg pPIV3-P and 0.1 µg pPIV3-L. Under these conditions approximately 1000 pfu per 6 X 10^5 cells were obtained during the initial transfection and 10^6 pfu per 6 x 10^5 cells after the amplification.

When individual plasmids were omitted from the transfection step it was observed that the pHPIV-3, pPIV3-P and pPIV3-L plasmids were required for recovery of virus but, surprisingly, as shown in Table 1 below, pPIV3-NP was not.

Table 1

Plasmids Transfected				
HPIV-3	NP	P	L	Virus Recovery
+	+	+	+	14/15[a]
-	+	+	+	0/2
+	-	+	+	3/3[b]
+	+	-	+	0/3
+	+	+	-	0/2

Table 1. Recovery of HPIV-3 from pHPIV-3. HeLa cell monolayers were infected with vTF7-3 and transfected with the indicated plasmids. After 40 hr cells were lysed and supernatants added to fresh HeLa cell monolayers in the presence of araC to inhibit vTF7-3 replication. These monolayers were then lysed

and the supernatants assayed for HPIV-3. The number of experiments for which HPIV-3 was recovered per attempts is displayed under virus recovery.

[A]The single experiment that did not yield HPIV-3 used 0.1 ug of the P plasmid.

[B]The omission of the NP plasmid resulted in 3 to 5 fold lower titers of HPIV-3.

Characterization of Recovered Virus.

In order to characterize the recovered virus and to purify HPIV-3 from vTF7-3, plaques of which are only slightly smaller than those of HPIV-3, isolated plaques suspected to be HPIV-3 were picked and amplified in HeLa cells. The plaque purified and amplified virus isolates and appropriate controls were then analyzed in neutralization assays. Virus (isolates #3 and #5) was preincubated (30 min on ice) with 5 ul rabbit pre-immune serum, 5 ul rabbit polyclonal anti-HPIV-3 antisera, or assayed in the presence of 25ug/ml araC The sera was incubated with virus on ice for 30 min prior to a standard plaque assay. To allow maximal plaque development, the plates were then incubated at 37°C for 66 hr prior to staining with crystal violet. The results of the assay indicated that the plaque purified virus was completely inhibited by the anti-HPIV-3 antisera, while vTF7-3 was not. In contrast, the HPIV-3 isolates were not inhibited by AraC, whereas the vTF7-3 virus was completely inhibited. Interestingly, of the eight recombinant HPIV-3 isolates, four had plaque sizes identical to the parental HPIV-3 stock while four were slightly larger. The plaque size of isolate #3 was slightly larger than isolate #5 and the wild type HPIV-3 virus.

To determine whether the NP coding sequence of pHPIV-3, which shares the same position as luciferase in pPIV3-MG(+), was being expressed from pHPIV-3, pHPIV-3 and pPIV3-NP were separately transfected into vTF7-3 infected HeLa cells and cell lists prepared after 48 hr. The lysates were then analyzed by Western blotting using an anit-HPIV-3RNP antisera. This antisera recognizes primarily NP and reacts poorly with P.

Specifically, extracts (equivalent to $6x10^4$ cells) were run on SDS-10% PAGE and transferred to nitrocellulose membranes. The primary antibody was a rabbit polyclonal anti-RNP antisera diluted 1:1000. The secondary antibody was 1:1000 dilution of a goat anti-rabbit antibody conjugated to horseradish peroxidase. Visualization was through chemiluminescence (ECL kit, Amersham). As shown by the Western blot, HPIV-3 RNP recognized NP from the pPIV3-NP and pHPIV-3 transfected cell extracts and from purified HPIV-3 RNP. No proteins were recognized in a mock-transfected HeLa extract. Thus, it appears that NP is expressed from pHPIV-3, presumably being translated from the T7-directed, antigenomic RNA transcript.

To determine whether the recovered, recombinant virus had specific mutations in its genome, RNA was extracted from wild type and plaque isolated viruses and used for RT-PCR analysis using primers flanking the substitution mutations. Viral RNA was isolated from approximately $2x10^7$ plaque forming units (pfu) of plaque purified virus isolates #3, 5, 7 and 9, or wt HPIV-3 strain 47885/. Reverse transcription was carried out using Superscript II reverse transcriptase (BMB) at 44°C for 1 hr using

oligonucleotides which primed at viral base 23 or 15100. The PCR was carried out with Expand Long polymerase (BMB) using second primers which result in amplification of viral bases 23 to 303, or 15100 to 15440.

PCR products encompassing viral bases 1 to 324 and 15080 to 15462 were generated from the indicated isolates, digested with SacI and StuI, respectively, and analyzed on a 1.4% agarose gel.. PCR products of the expected sizes were generated in a RT-dependent manner, indicating that the PCR products were derived from RNA rather than contaminating plasmid DNA.

As shown on the agarose gel, the sizes of the 1 to 324 and 15080 to 15462 PCR products are increased by 21 and 22 base pairs, respectively, over the length of the viral specific regions due to the inclusion of restriction enzyme sites in the amplification primers. Digestion with SacI showed that the mutation at base 94 was not present in the wild-type virus but was present in the plaque isolated viruses, indicating they are of recombinant origin. Similarly, PCR product derived from the region encompassing viral base 15389 of wild type HPIV-3 was not cleaved by StuI. However, only four of the eight plaque isolated viruses contained the mutation which creates the StuI site. Direct sequencing of the PCR products confirmed these results.

DISCUSSION

A full-length plasmid clone of the HPIV-3 genome, pHPIV-3 was constructed.. Upon transfection of pHPIV-3 and plasmids encoding the viral NP, P and L proteins into vTF7-3-infected HeLa cells, recombinant HPIV-3 bearing genetic markers was efficiently recovered. Several interesting features of this system were noted. First, the viral NP protein could be expressed from the infectious clone, and this expression obviated the need for an NP support plasmid. It is believed that the NP protein is synthesized directly from the primary anitgenomic transcript after it is capped by the vaccinia virus capping enzyme.

Second, two recombinant viruses with distinct genotypes and phenotypes were produced, probably due to recombination between the pHPIV-3 and pPIV3-L plasmids, although the possibility that the reversion arises during RNA replication cannot be excluded. pPIV3-L contains the entire L 3' UTR and part of the trailer region, extending to base 15437, an overlap of 48 base pairs beyond the StuI site. This is ample room for recombination between plasmids to occur readily in vaccinia virus infected cells

From these results, there appears to be selection in the HPIV-3 system. All the large plaque virus isolates had reverted to a wild-type sequence at base 15389, while retaining the change at base 94. Since A94G is the only known alteration in these viruses from the parental virus, it appears that The isolates which retained both mutations had a plaque size identical to that of the parental (wild type) virus, but when the 15389 mutations was lost, plaque size increased, indicating that the mutation at base 15389 was detrimental in the context of the A94G mutation. There was one other known change between pHPIV-3 and the support plasmids. A non-initiating AUG exists in the natural L protein message. Since this AUG is only 11 nucleotides from the 5' end of the L mRNA and in a poor translation initiation context, it may not cause much interference with L mRNA translation. However, in the support plasmid pPIV3-L this

non-initiating AUG is much further from the 5' end of the transcript where it is more likely to be recognized by ribosomes. This AUG was removed from pPIV3-L by changing bases 8636 and 8637 from AT to TA, destroying a SphI site and creating a NheI site. To investigate whether this change was present in the recombinant virus and could be responsible for the large plaque phenotype, RT-PCR analysis was done. PCR products encompassing this site and derived from both the wild type and the plaque isolated viruses retained a wild type sequence, indicating recombination had not occurred over this region and that these changes could not account for any variance in plaque size.

The finding that recombination may occur between transfected plasmids indicates that care must be taken when introducing mutations into the paramyxovirus or rhabdovirus infectious clone systems. Mutations introduced within the NP, P or L sequences preferably are carried by both the support plasmids and the infectious clone. Otherwise, resultant virus may not carry the desired mutation. The only possible exception to this is the HPIV-3 system, in which the HPIV-3 infectious clone expresses NP, negating the need for the NP support plasmid. Still, it is preferred that the HPIV-3 NP support plasmid be included in the system, since significantly greater yields of HPIV-3 were obtained when the support NP plasmid was included in the transfection.

An infectious clone for HPIV-3 is useful for understanding the molecular biology of HPIV-3 and for developing a vaccine for this important pathogen. The ability to generate specific mutations within HPIV-3 makes all aspects of HPIV-3 replication amenable to study. Any mutation, including those studied previously in other contexts, can now be examined with this system. The ability to introduce specific mutations also permits the possibility of revertant analysis, which could refine our understanding of protein-protein or protein-RNA interactions.

The infectious clone is also useful for identifying mutations which attenuate the virus. Such virus is useful for developing new vaccine strains of HPIV-3. In addition, mutations present in a current candidate vaccine strain of HPIV-3 can be inserted into pHPIV-3. Through identifying multiple deleterious mutations, it should be possible to engineer several mutations affecting various steps in the virus life cycle into a single HPIV-3 strain. Such a virus should be highly attenuated and not readily able to revert.

While the invention has been described to some degree of particularity, various adaptations and modifications can be made without departing from the scope of the invention as defined in the appended claims

CLAIMS

What is claimed is:

1. A recombinant HPIV clone comprising:

a. a nucleotide sequence encoding a positive sense, antigenome of human parainfluenza virus; and

b. an RNA polymerase promoter operatively linked to said nucleotide sequence.

2. The clone of claim 1 wherein the nucleotide sequence encodes the antigenome of HPIV-3.

3. The clone of claim 2 wherein said clone further comprises a ribozyme sequence downstream from said antigenome-encoding sequence.

4. The clone of claim 2 wherein the RNA polymerase promoter is the T7 RNA polymerase promoter.

5. The clone of claim 3 wherein the ribozyme is an antigenomic ribozyme.

6. The clone of claim 3 further comprising an RNA polymerase terminator downstream of the ribozyme sequence.

7. The clone of claim 2 wherein the nucleotide sequence encodes a modified anti-genome of HPIV-3.

8. The clone of claim 2 wherein the clone has the characteristics of a plasmid deposited with the American Type Culture Collection and having Accession Number _____ .

9. The clone of claim 7 wherein the HPIV-3 antigenome-encoding sequence comprises a mutation selected from the group consisting of a substitution of one or more nucleotides, a deletion of from 3 to 12 nucleotides, and an addition of from 3 to 12 nucleotides.

10. A method for preparing recombinant human parainfluenza virus comprising;

a. providing a recombinant system which comprises:

i. an HPIV clone comprising a nucleotide sequence encoding a positive sense, antigenome of human parainfluenza virus;

ii a support clone comprising a nucleotide sequence encoding a human parainfluenza virus L protein; and

iii. a support clone comprising a nucleotide sequence encoding a human parainfluenza virus P protein,

wherein the nucleotide sequence encoding the P protein and L protein may be on the same support clone or on separate support clones;

b. introducing the recombinant system of step (a) into host cells;

c. culturing the host cells of step (b) for a time sufficient to permit transfection of the host cells and formation of recombinant human parainfluenza virus; and

d. recovering the recombinant human parainfluenza virus from the culture of transfected host cells.

11. The method of claim 11 wherein the antigenome-encoding sequence of the HPIV clone is operatively-linked to an RNA polymerase promoter;

wherein the P protein-encoding sequences of the support clones is operatively-linked to an RNA polymerase promoter ;

wherein the L protein-encoding sequences of the support clones is operatively-linked to an RNA polymerase promoter; and

wherein the host cells comprise an RNA polymerase corresponding to the RNA polymerase promoter of said HPIV clone and said support clones.

12. The method of claim 11, wherein the host cells are infected with a viral recombinant capable of expressing the RNA polymerase prior to or in combination with introduction of the recombinant system into the host cells.

13. A host cell for producing a recombinant human parainfluenza virus, said host cell comprising:

a. an HPIV clone comprising a nucleotide sequence encoding a positive sense, antigenome of human parainfluenza virus;

b a support clone comprising a nucleotide sequence encoding a human parainfluenza virus L protein; and

c. a support clone comprising a nucleotide sequence encoding a human parainfluenza virus P protein;

wherein the nucleotide sequence encoding the P protein and L protein may be on the same support clone or on separate support clones.

14. The host cell of claim 13 further comprising a support clone comprising a nucleotide sequence encoding a human parainfluenza virus NP protein.

15. The host cell of claim 13 wherein the HPIV clone comprises a nucleotide sequence encoding an anti-genome of HPIV-3.

16. The host cell of claim 15 wherein the anti-genome encoding sequence of HPIV-3 comprises a site-specific mutation.

17. The host cell of claim 14 wherein the HPIV-3 clone further comprises an RNA polymerase promoter operatively linked to the HPIV-3 antigenomic sequence and wherein each of the support clones comprises an RNA polymerase promoter operatively linked to the HPIV-3 protein-encoding sequence of said support clone.

18. A method of introducing a site-specific mutation into the genome of a recombinant human parainfluenza virus, comprising the following steps:

a. preparing a clone comprising a nucleotide sequence encoding a human parainfluenza viral antigenome having a mutation at a specifc site;

b. co-transfecting host cells with the clone of step (a),

a support clone comprising a nucleotide sequence encoding an HPIV L protein, and a support clone comprising a nucleotide sequence encoding an HPIV P protein;

wherein the nucleotide sequence encoding the P protein and L protein may be on the same support clone or on separate support clones; and

c. culturing the transfected host cells for a time sufficient to allow formation of a recombinant human parainfluenza virus.

19. The method of claim 18 further comprising the step of transfecting the host cells with a support clone comprising a nucleotide sequence encoding an HPIV NP protein.

20. The method of claim 18 wherein the clone of step (a) is prepared using polymerase chase reaction techniques and a clone comprising a nucleotide sequence encoding a human parainfluenza virus antigenome as a template, wherein the antigenome-encoding nucleotide sequence of said template clone lacks the site-specific mutation..

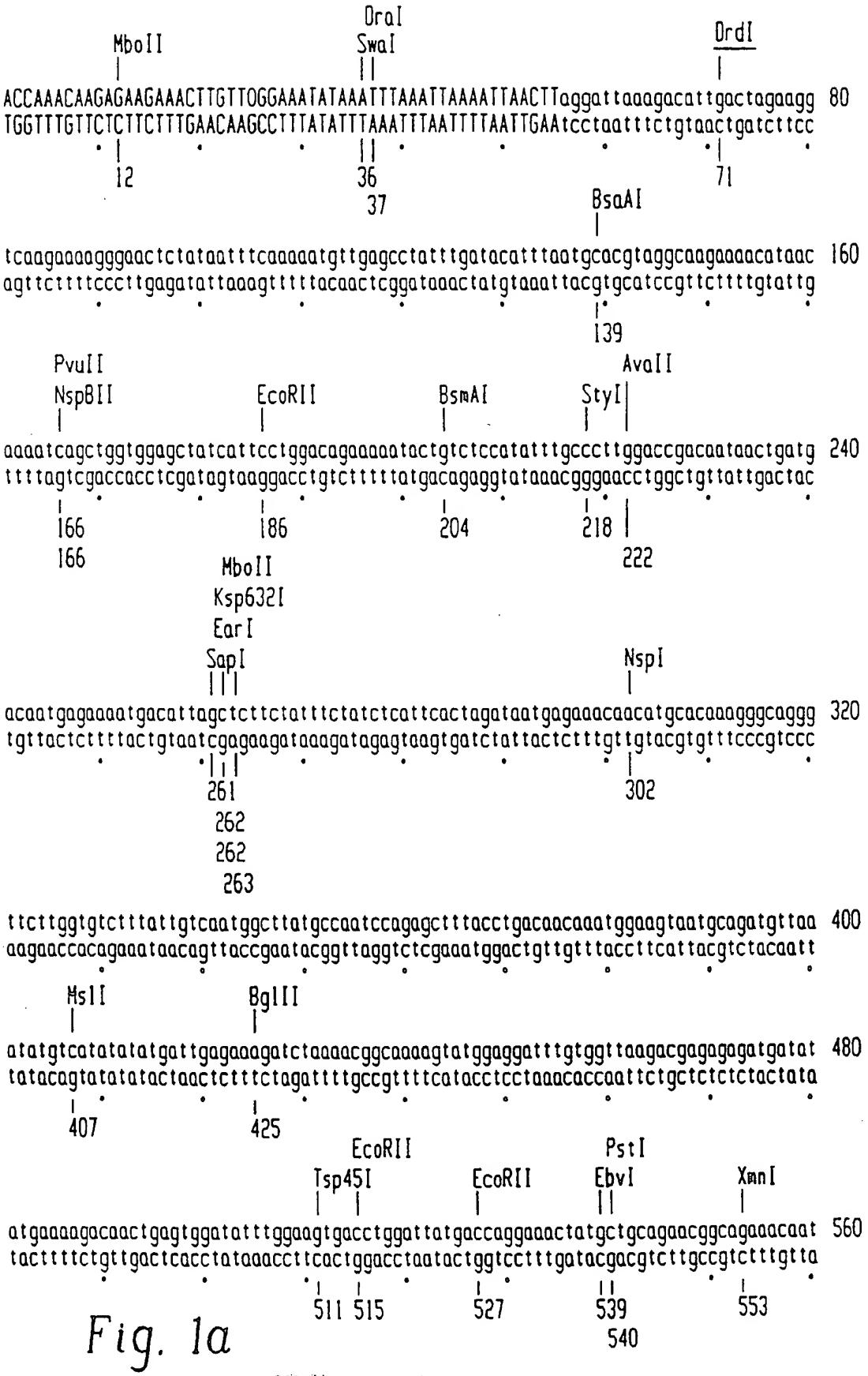
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Fig. 1a

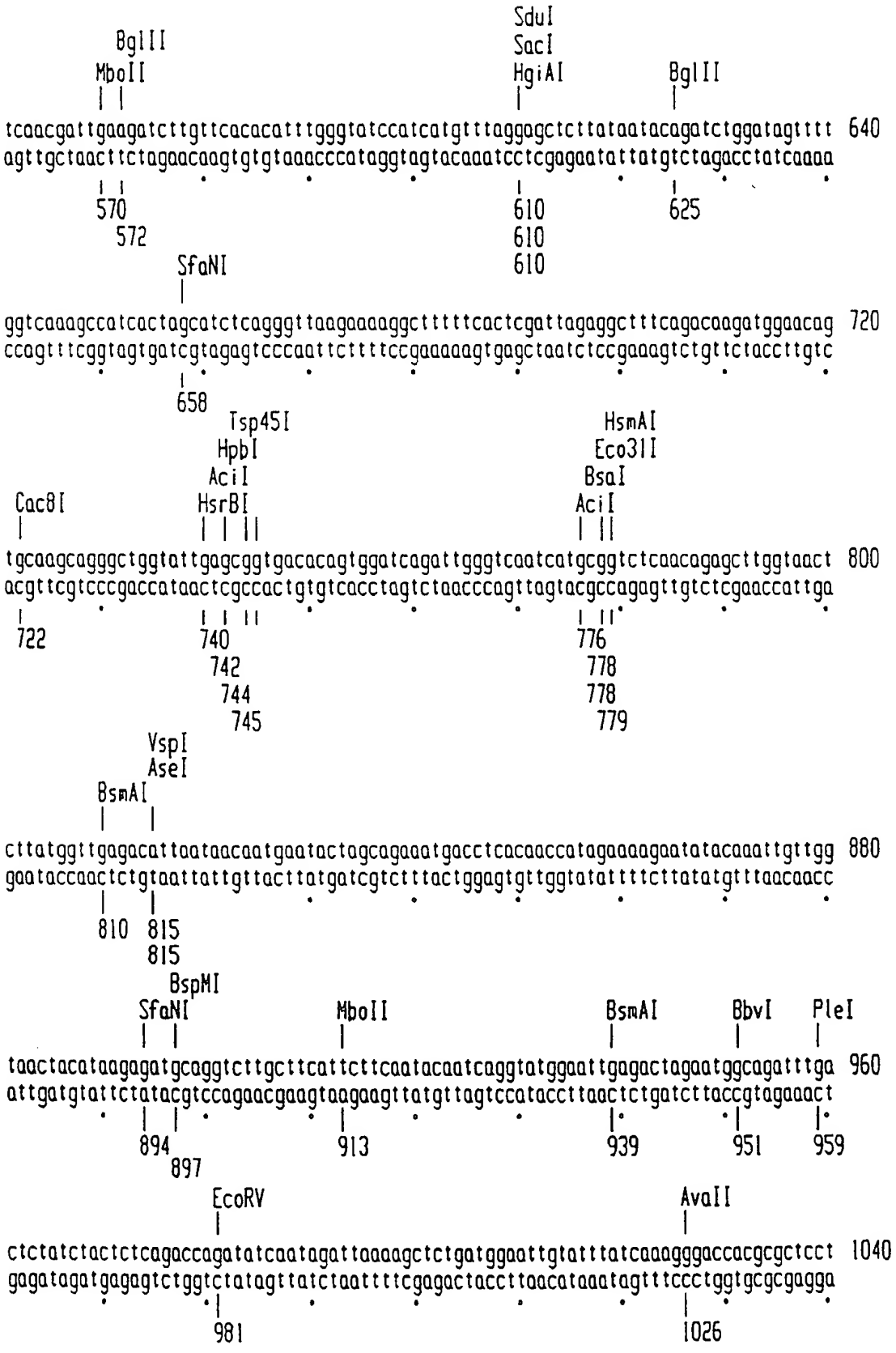
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Fig. 1b

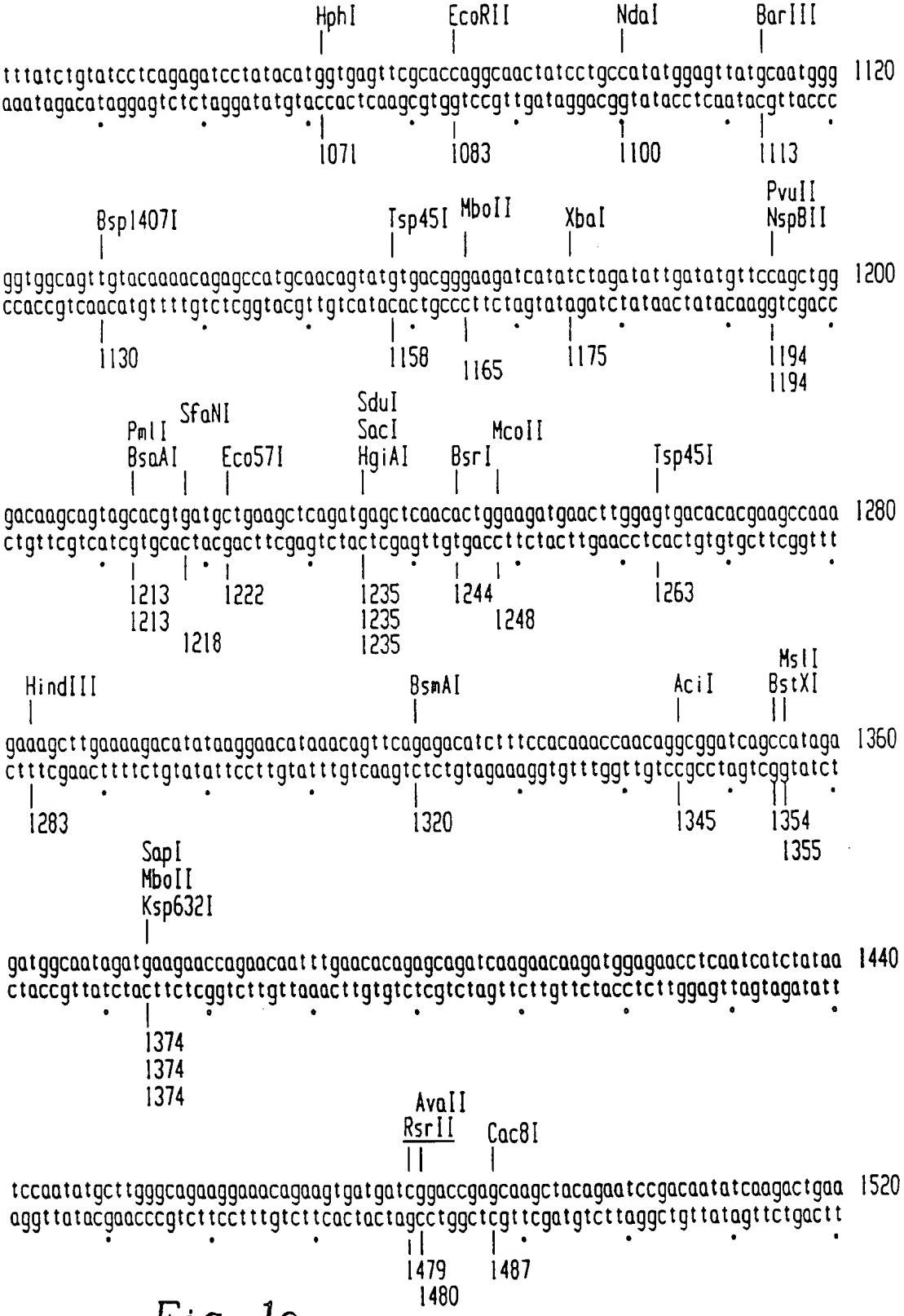
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Fig. 1c

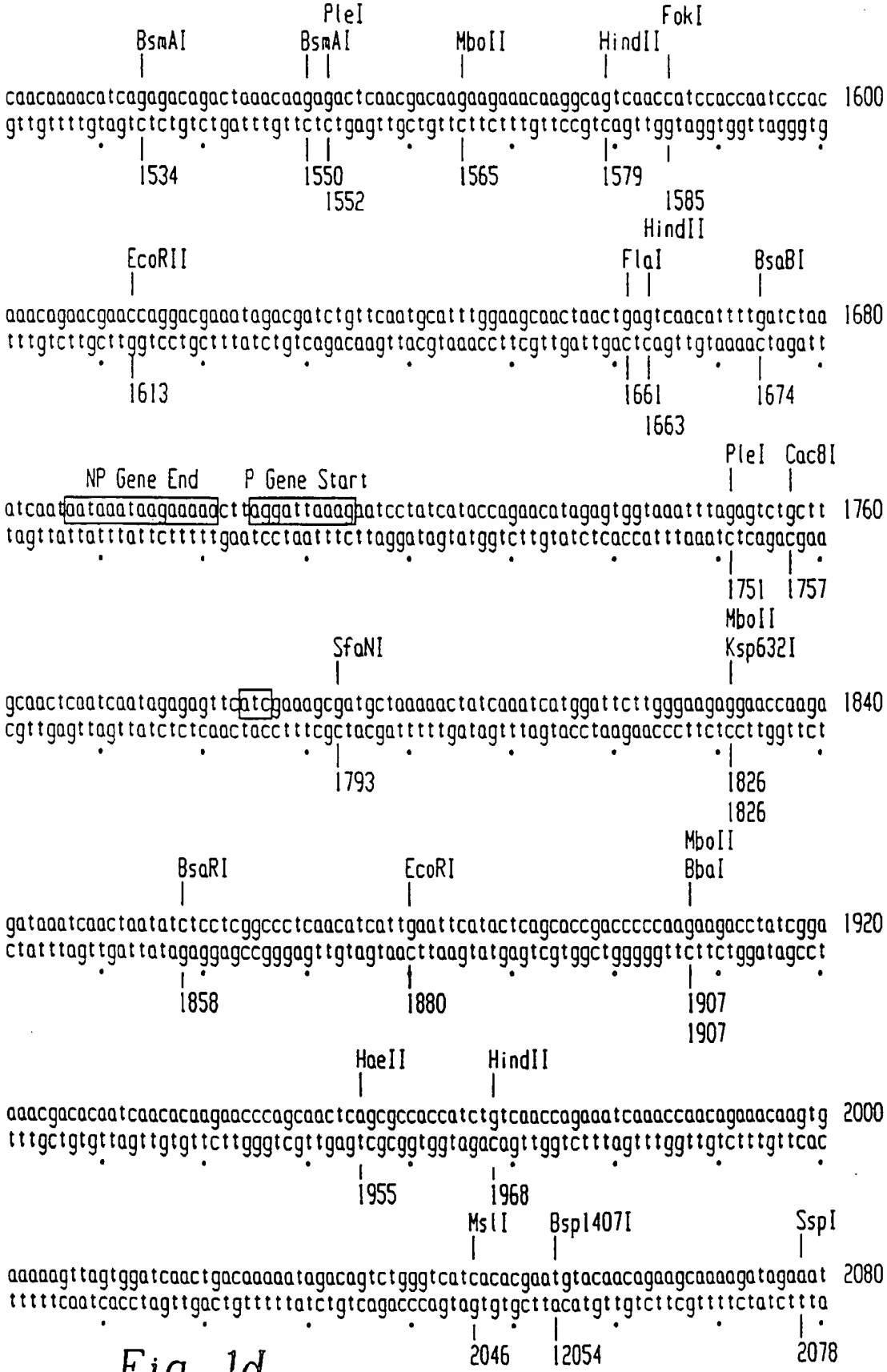
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Fig. 1d

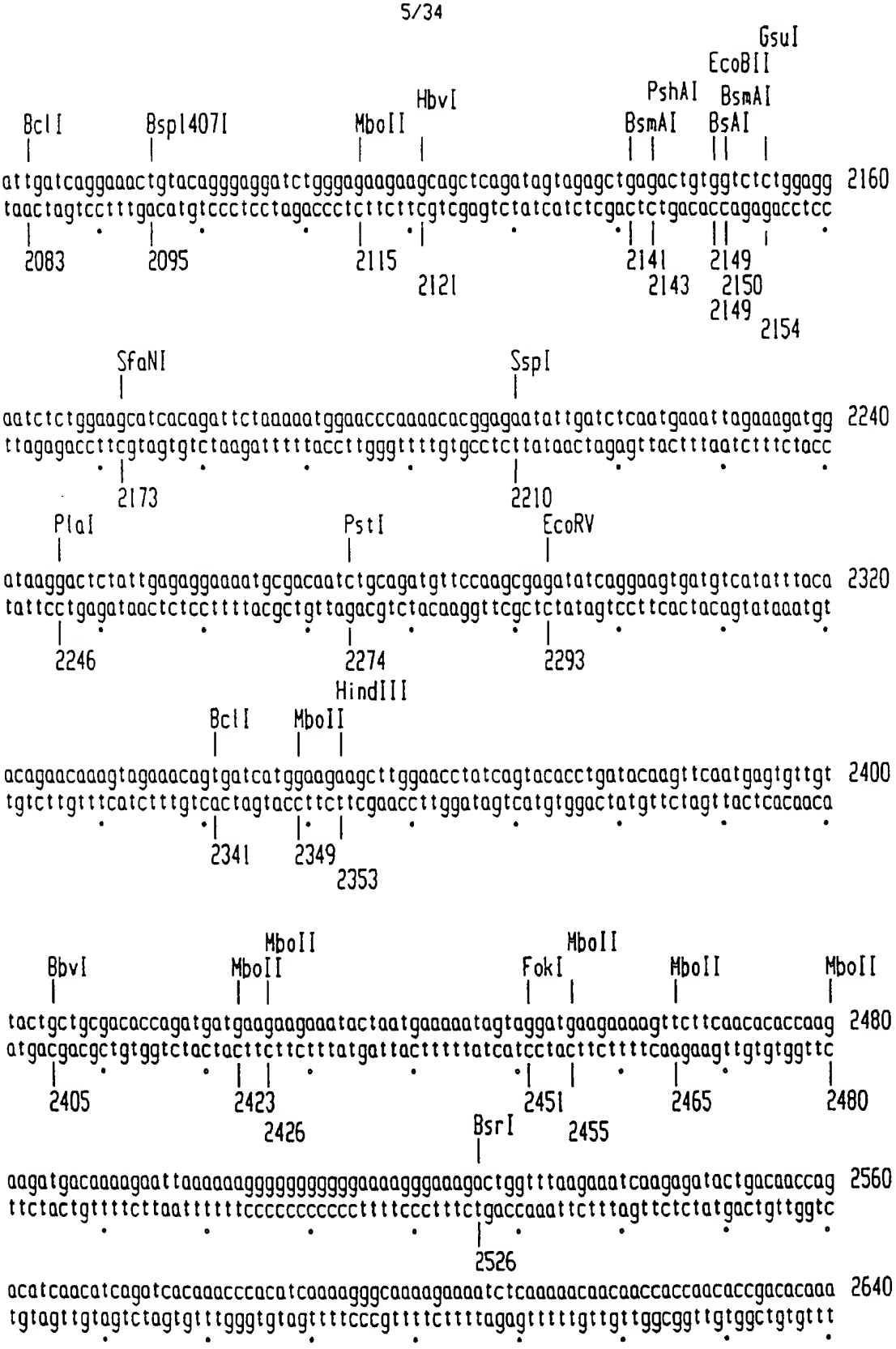
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Fig. 1e

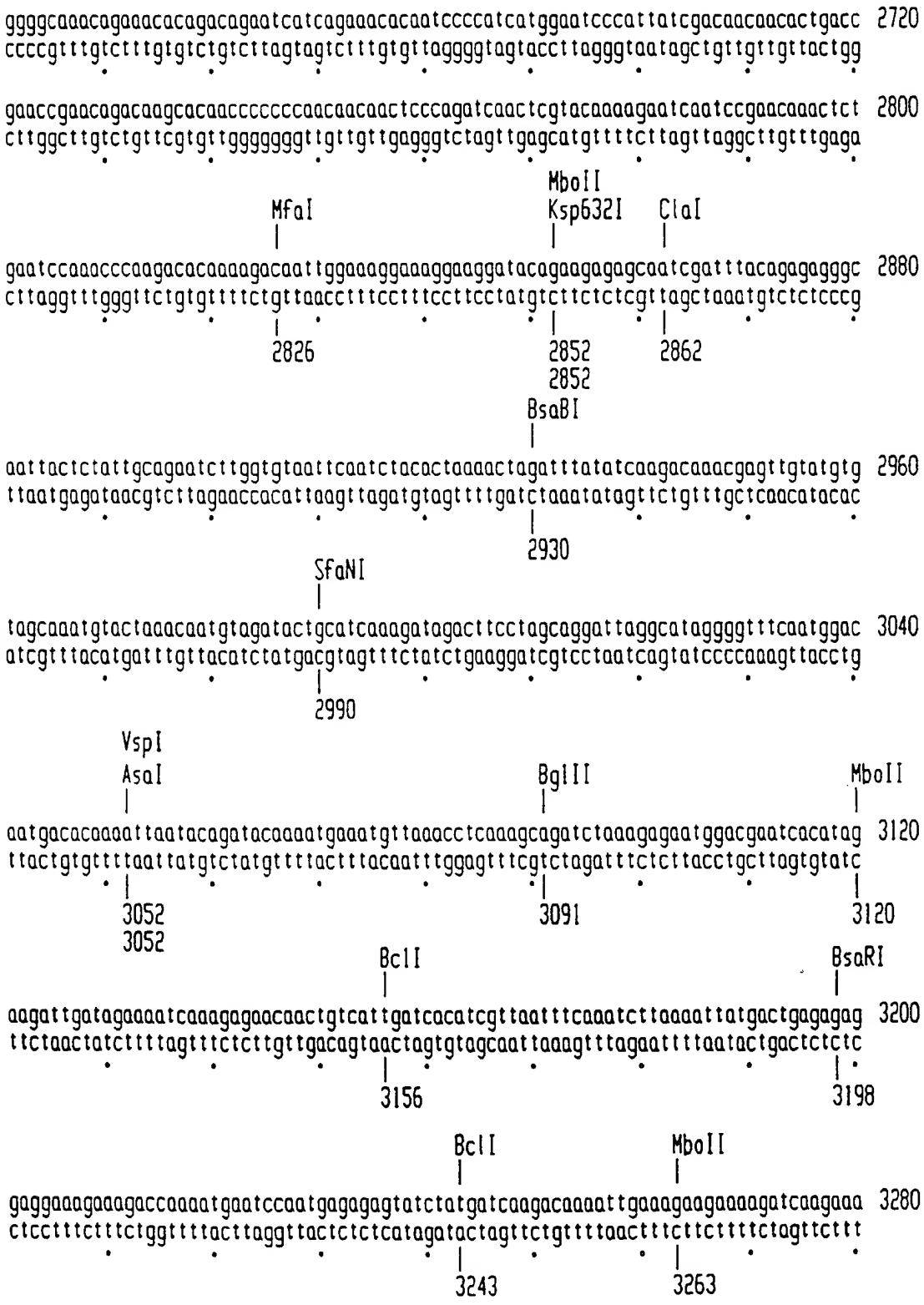
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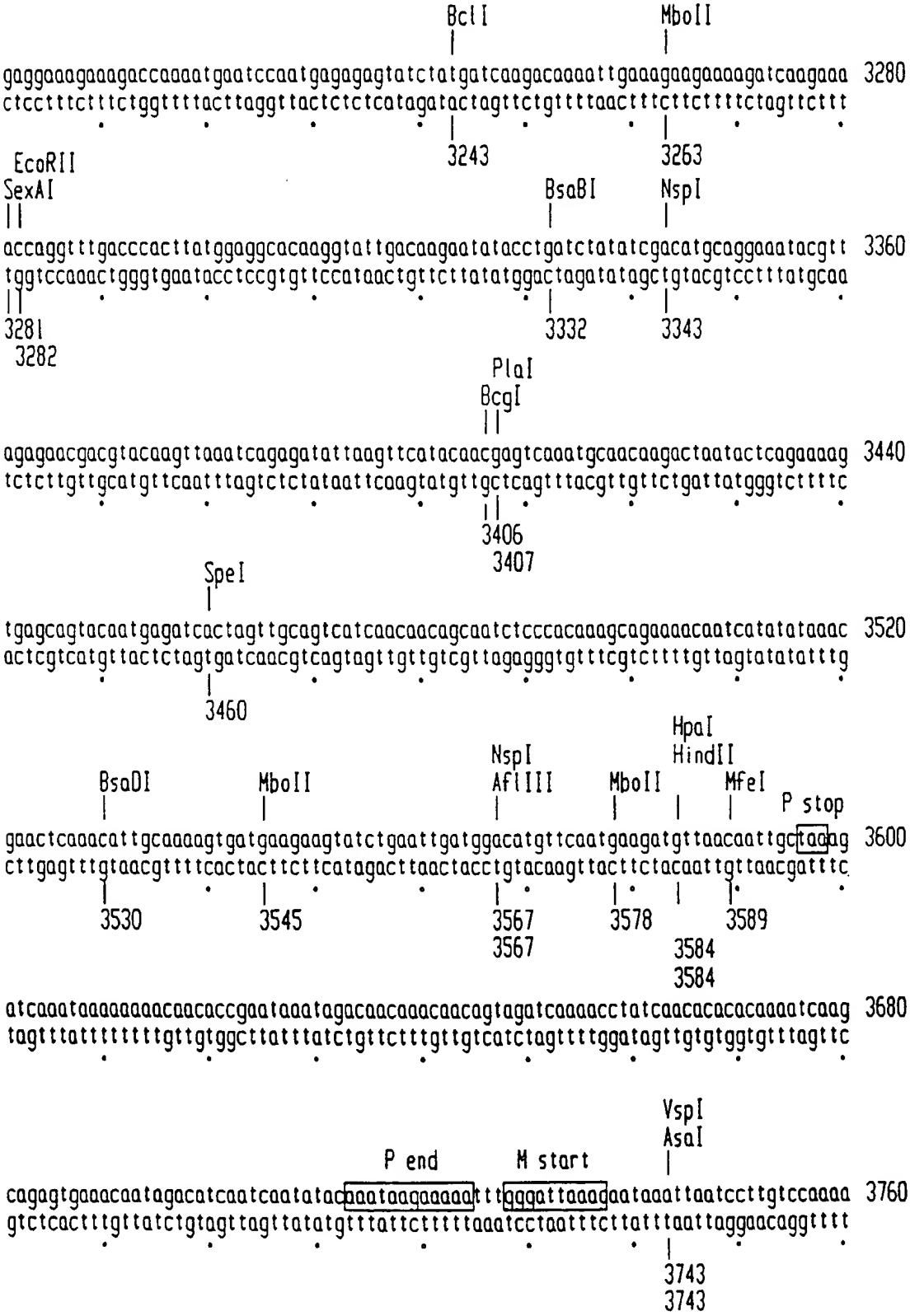
Fig. 1f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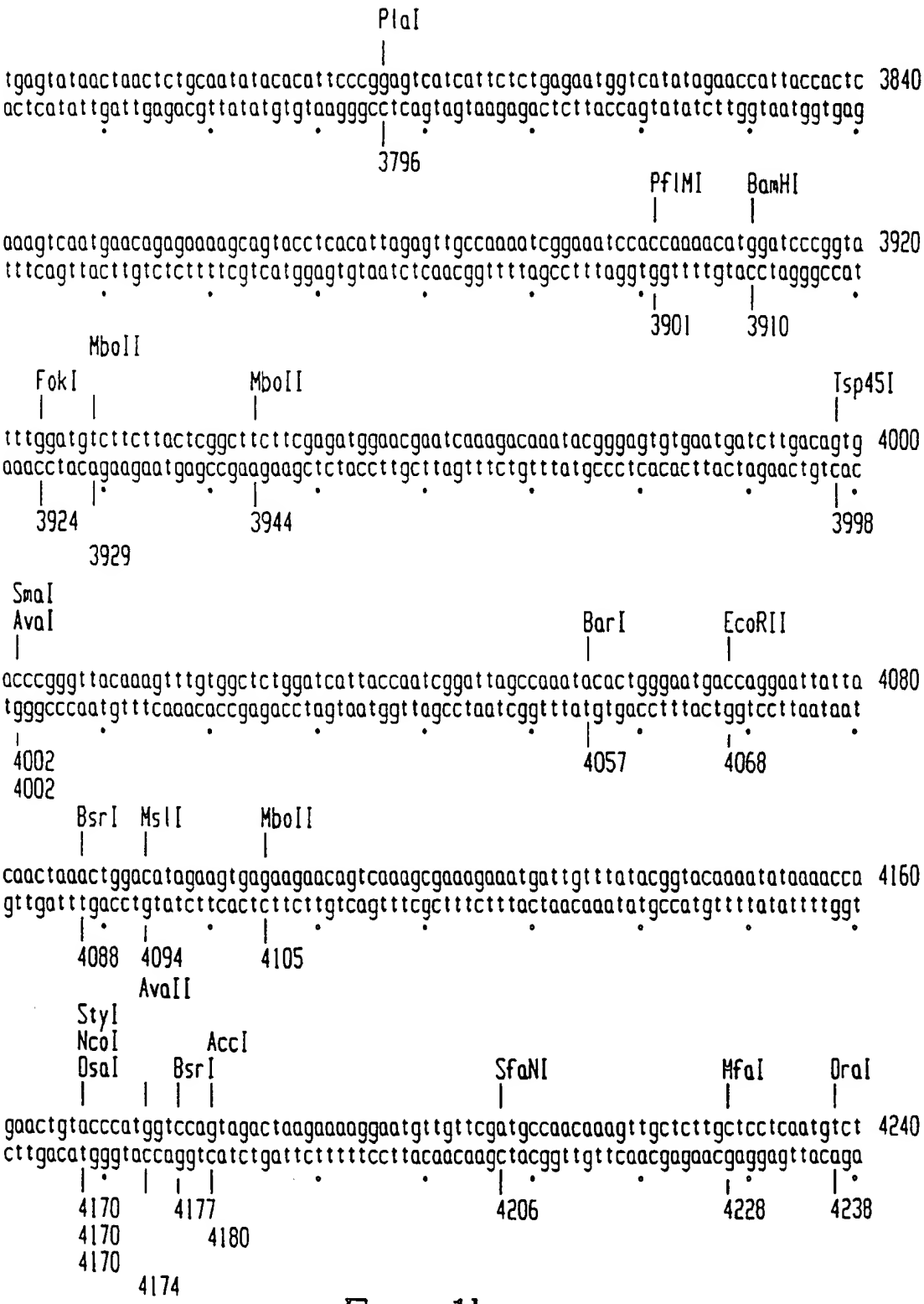
BsaDI
MboII
MboII
MfeI
P stop
VspI
AsaI
P end
M start

Fig. 1g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PflMI
BamHI
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MboII
FokI
MboII
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SmaI
AvaI
BarI
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AccI
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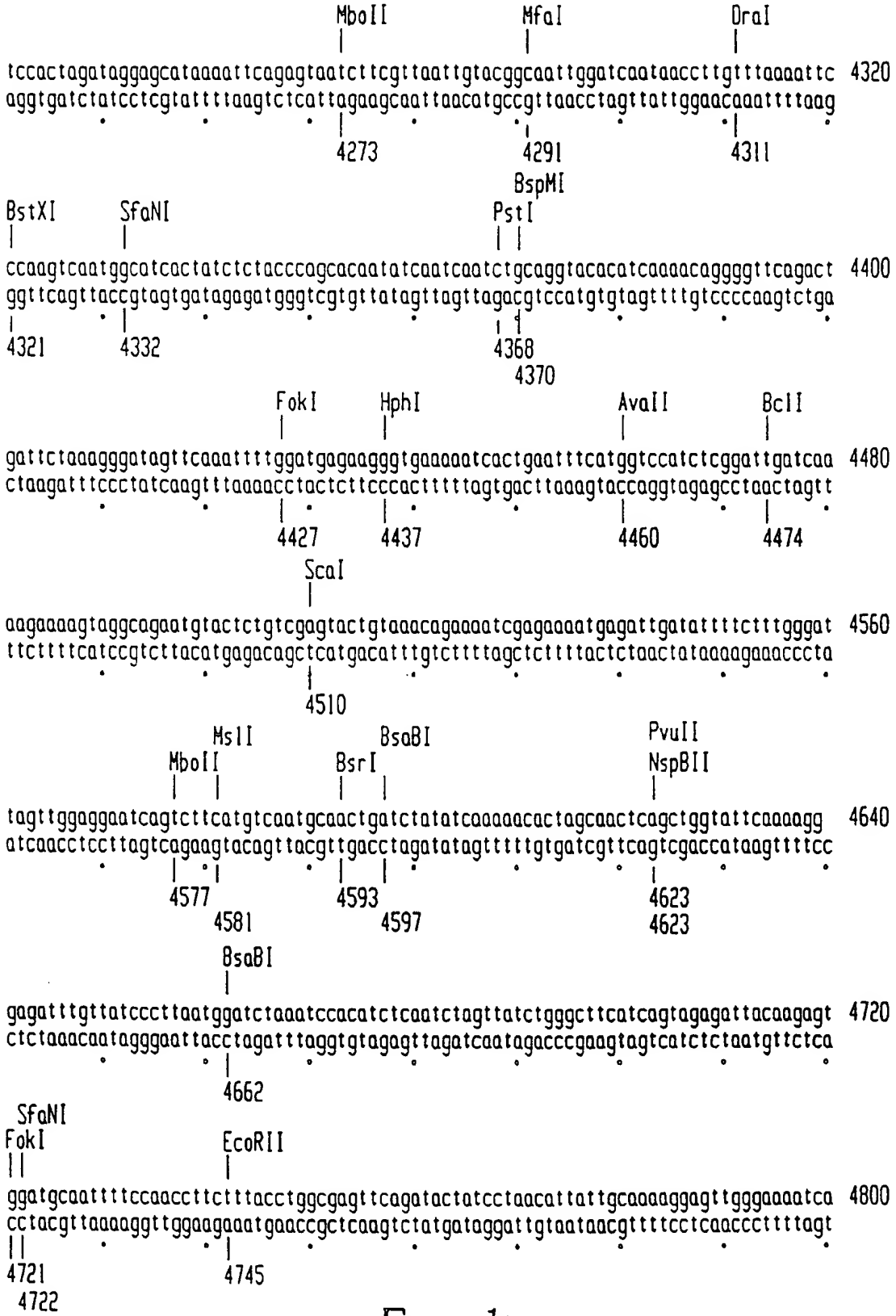
Fig. 1h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FokI
HphI
AvaII
BclI
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4437
4460
4474
ScaI
4510
MsII
BsaBI
PvuII
MboII
BsrI
NspBII
4577
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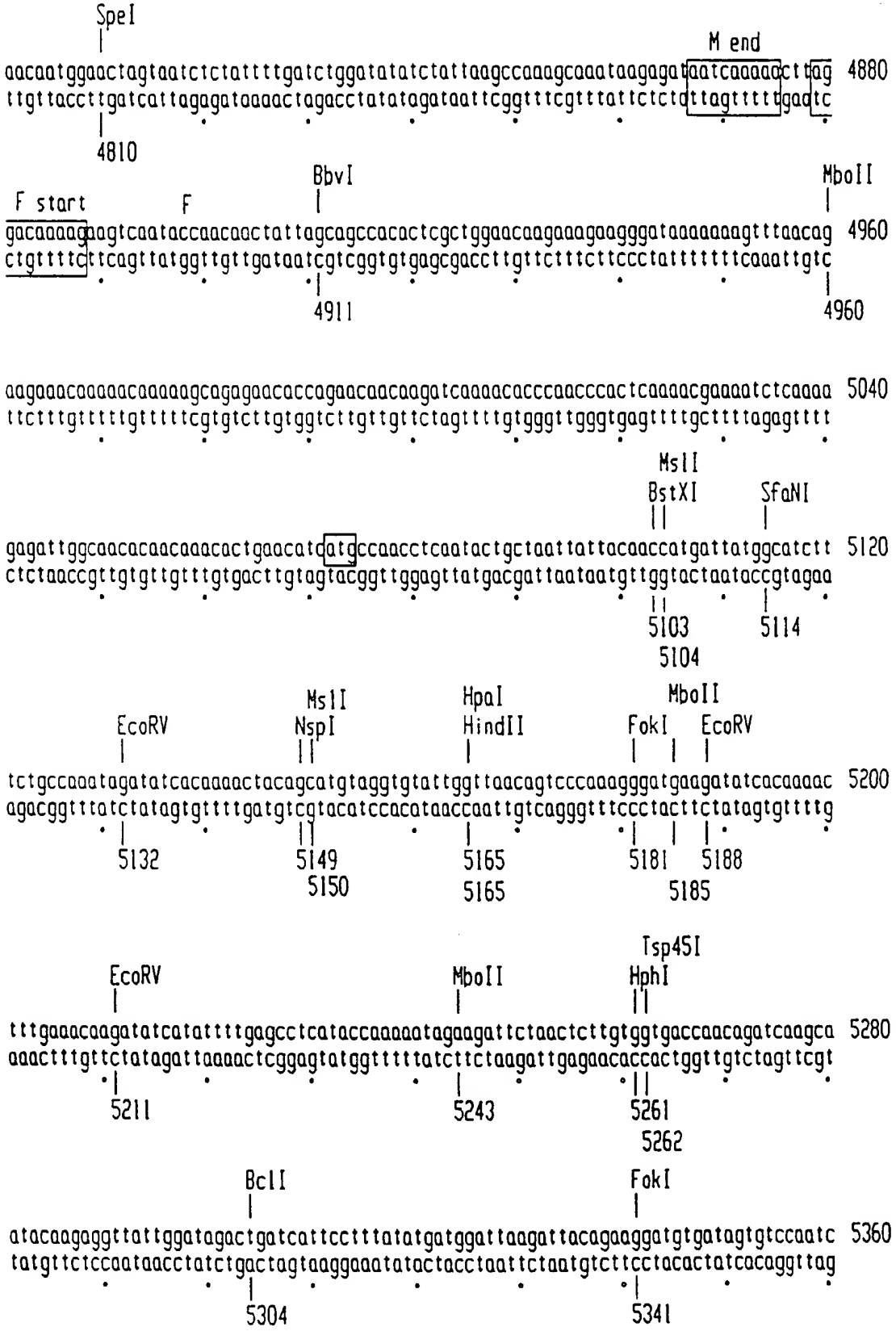
4722
4745

Fig. 1i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HindII
FokI
EcoRV
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Fig. 1j

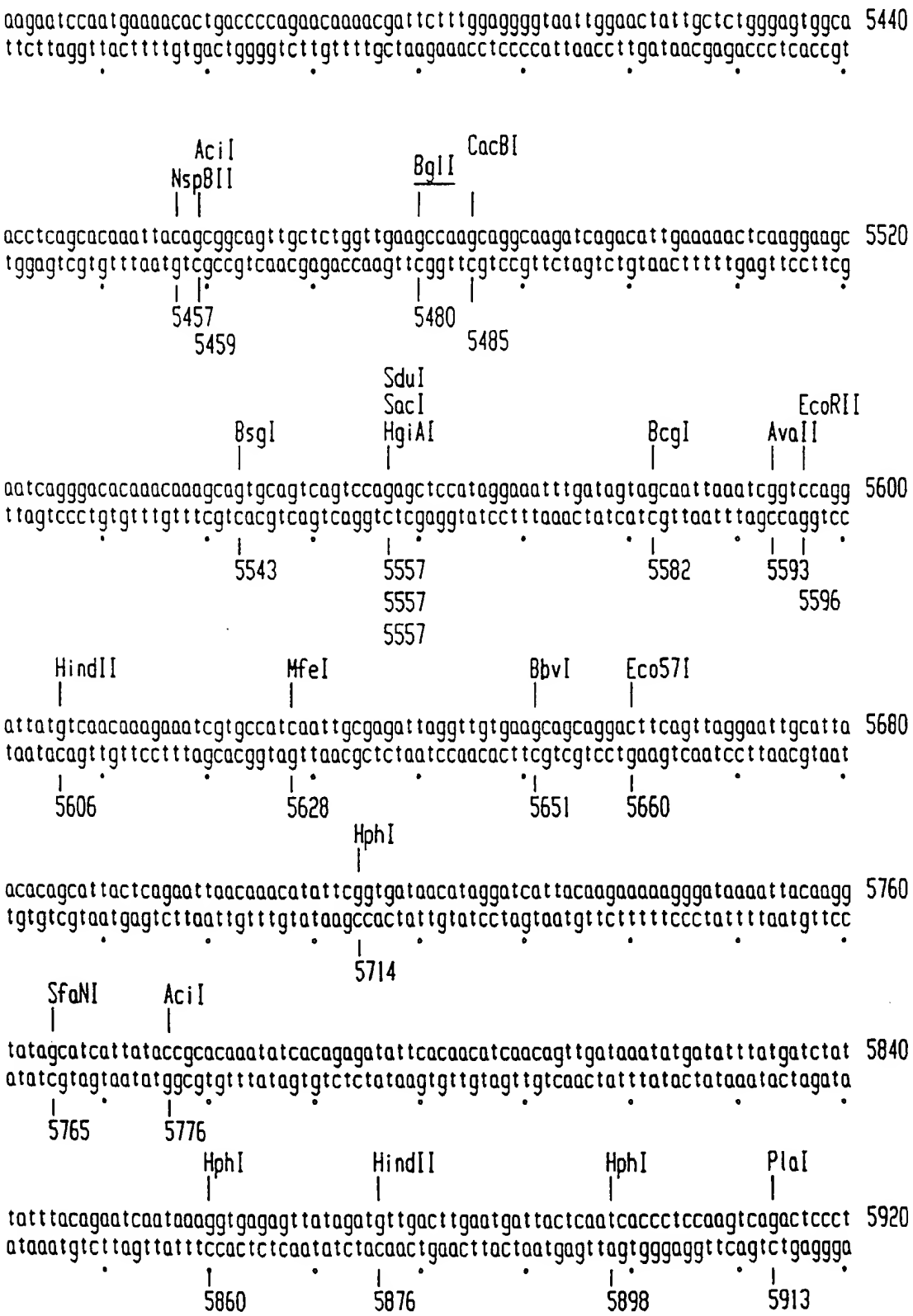
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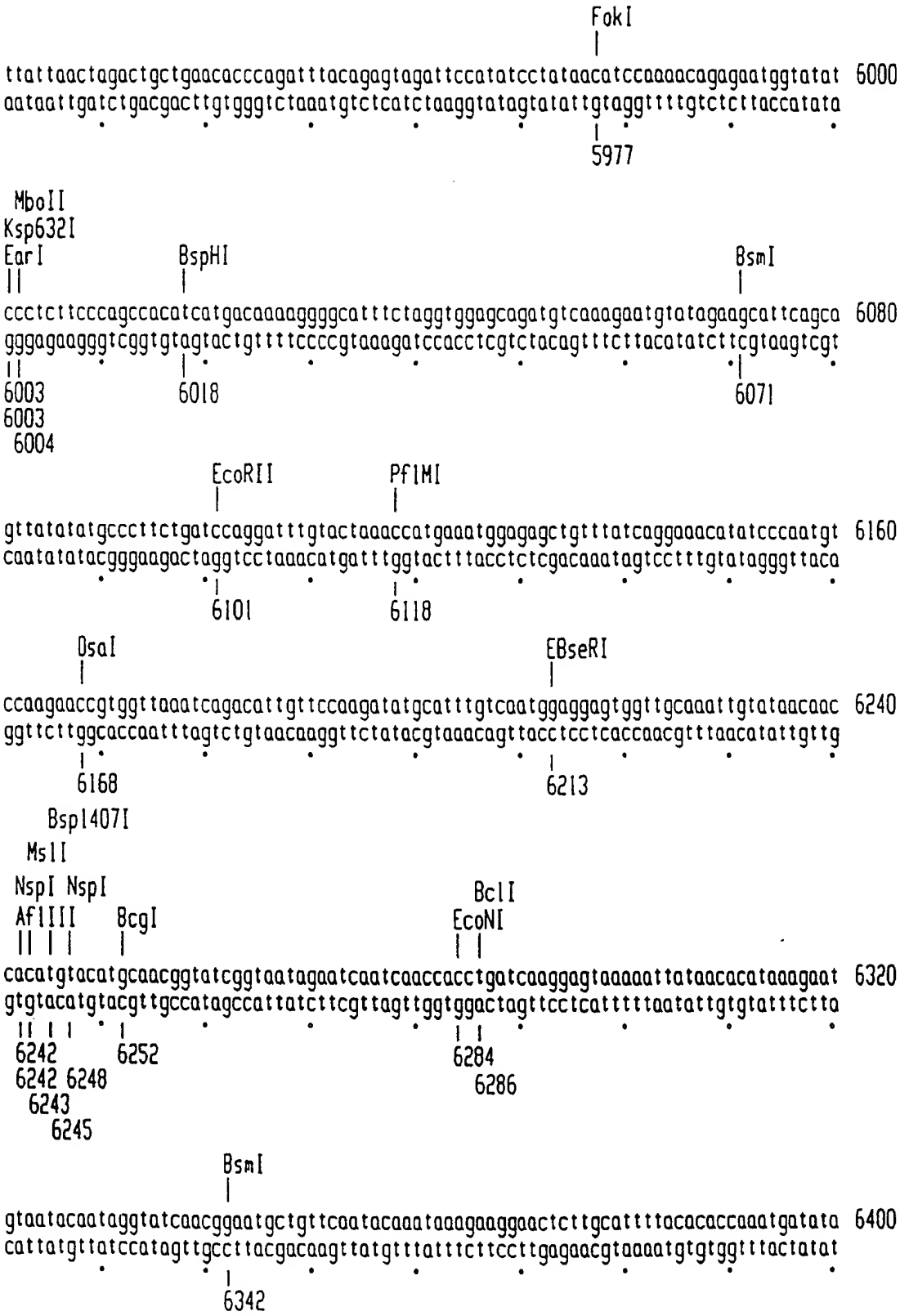
Fig. 1k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MslI
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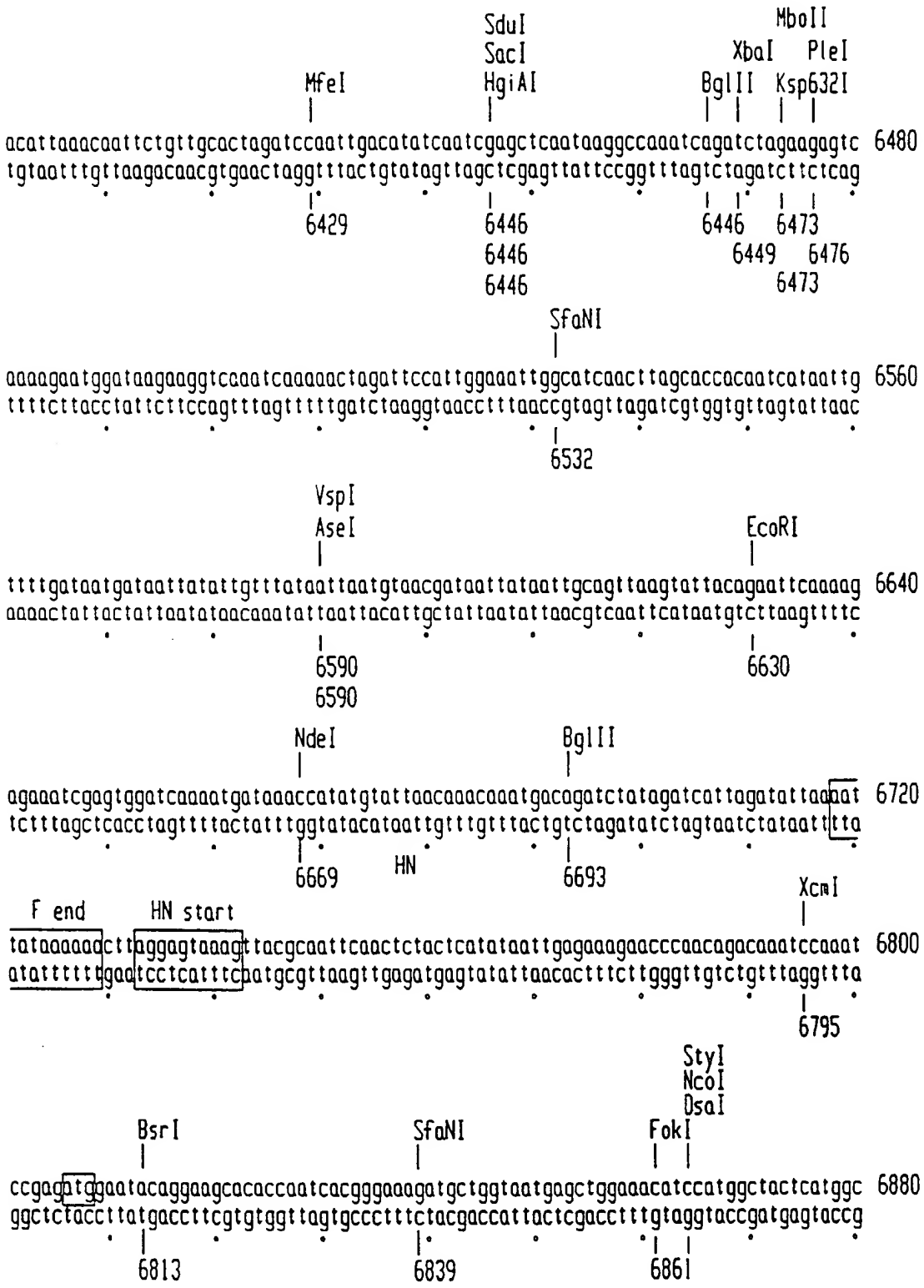
BsmI
6342

Fig. 11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EcoRI
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BglII
6669
HN
6693
XcmI
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6839
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Fig. 1m

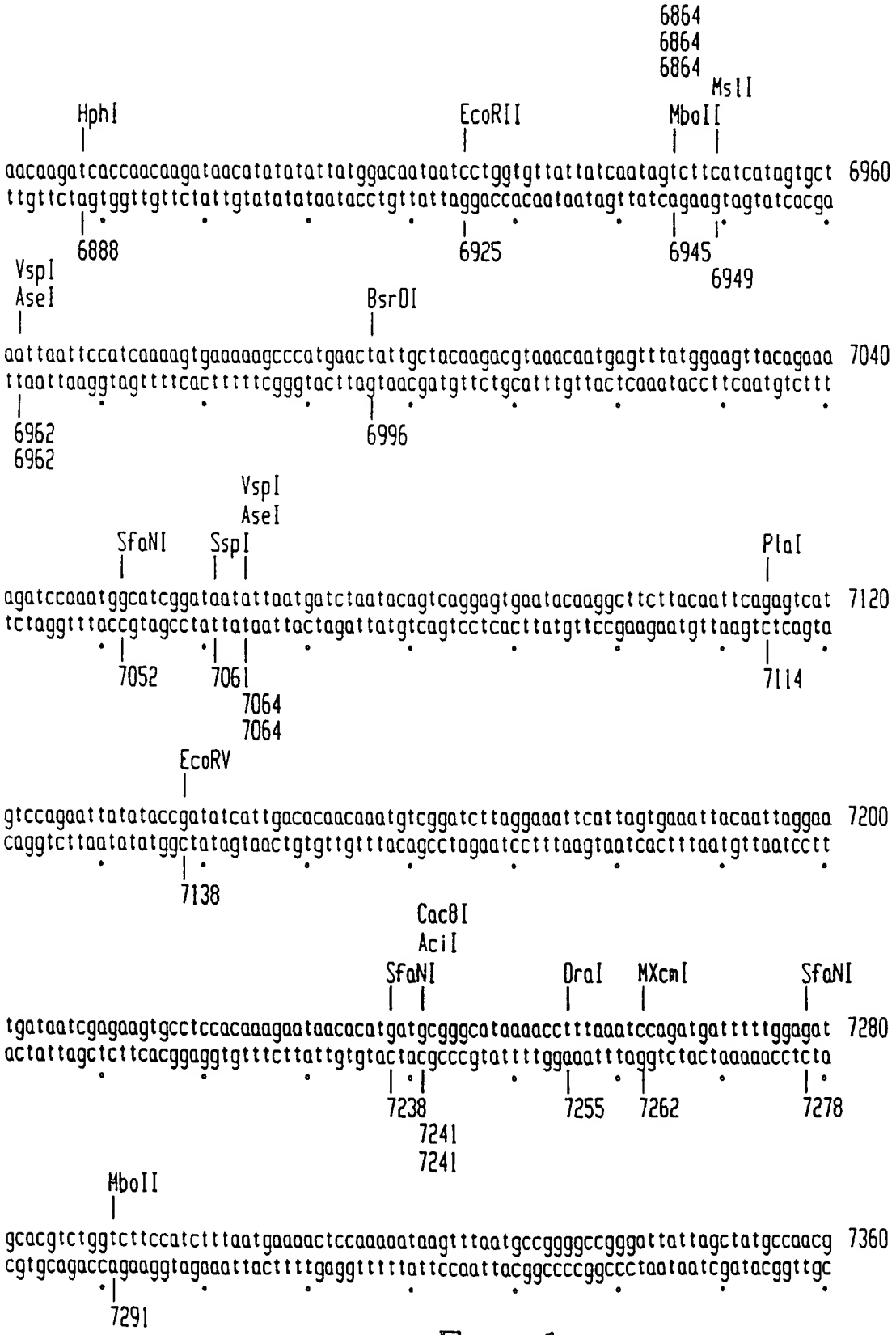
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Fig. 1n

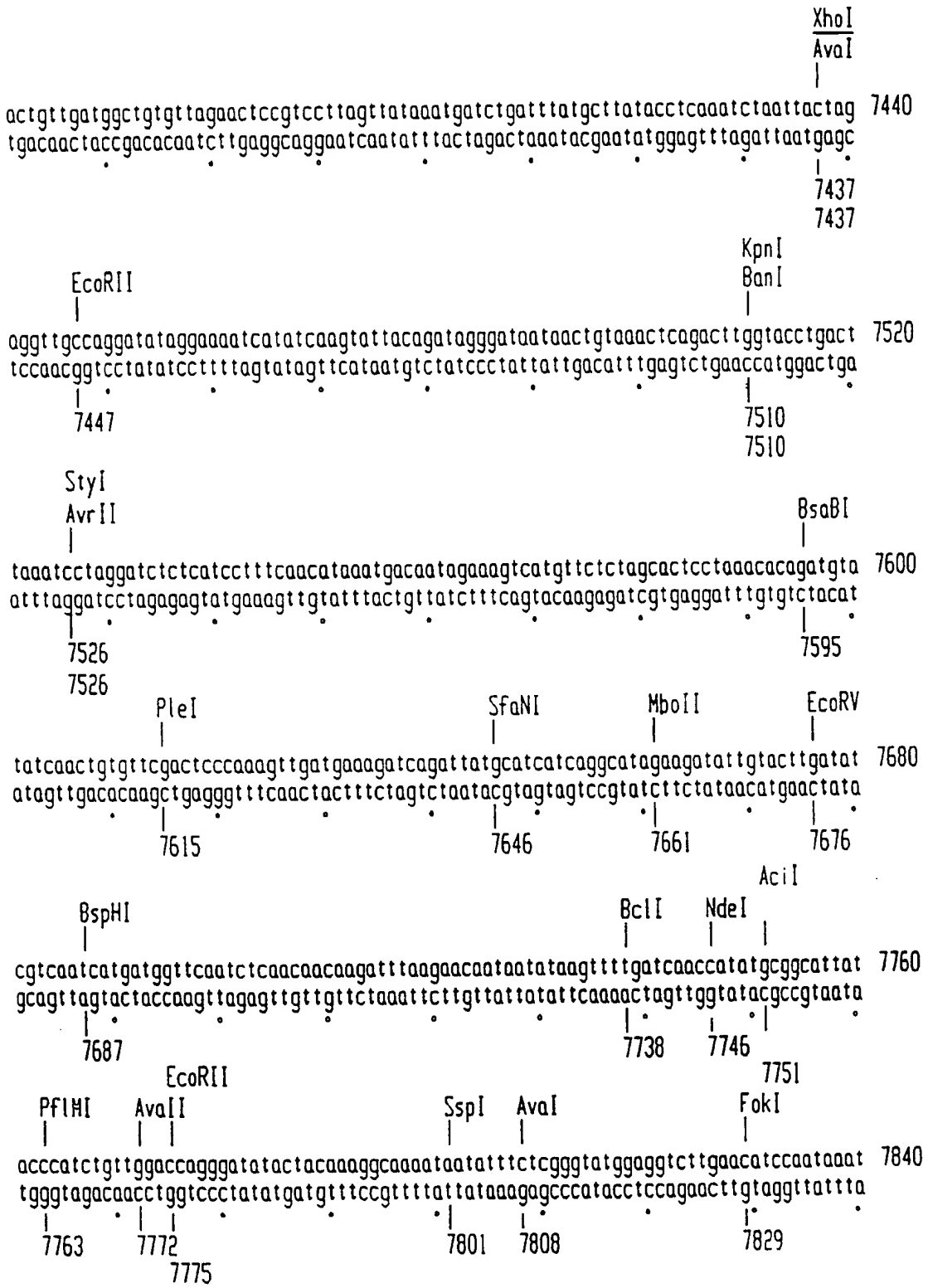
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Fig. 1o

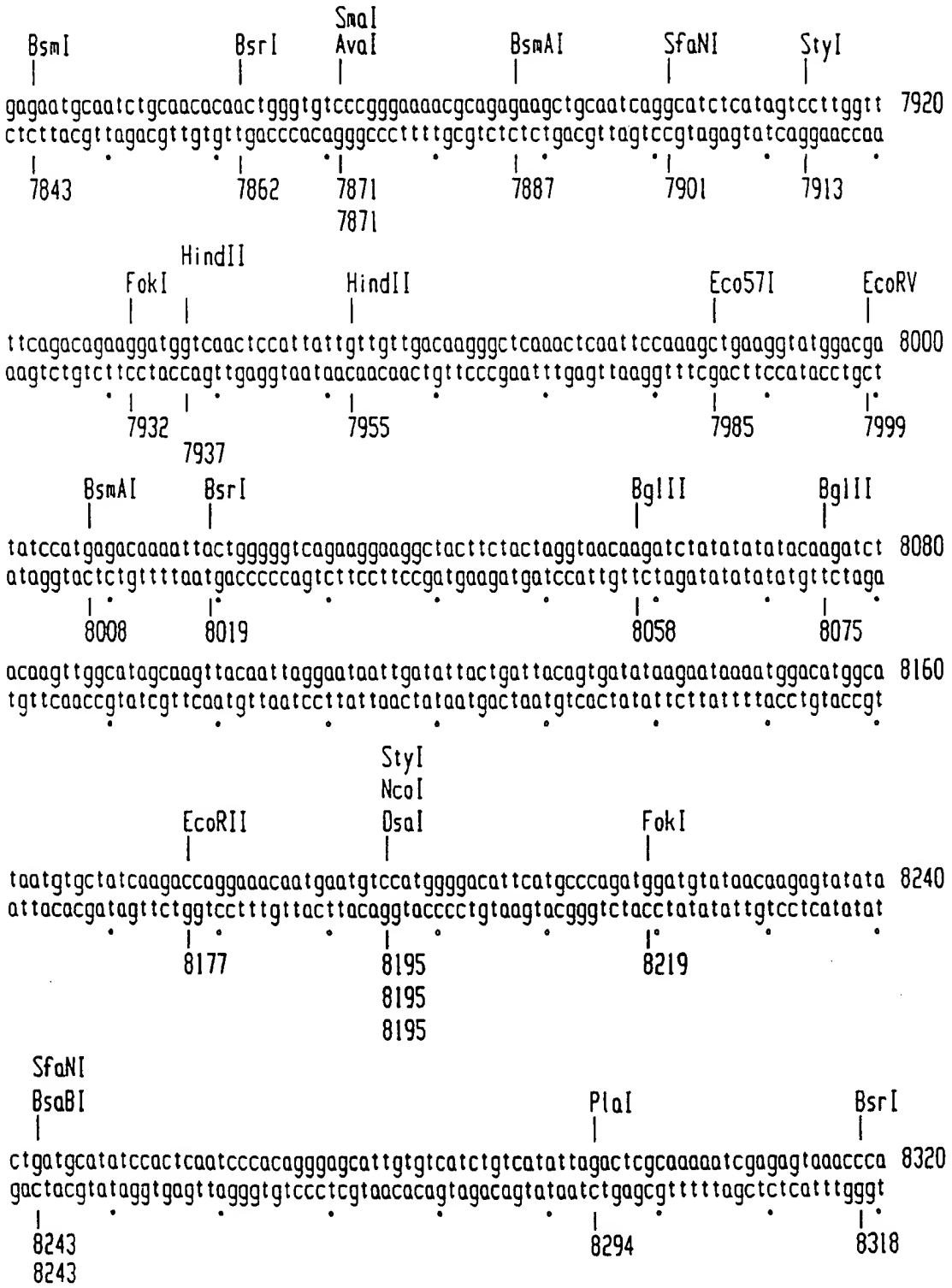
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Fig. 1p

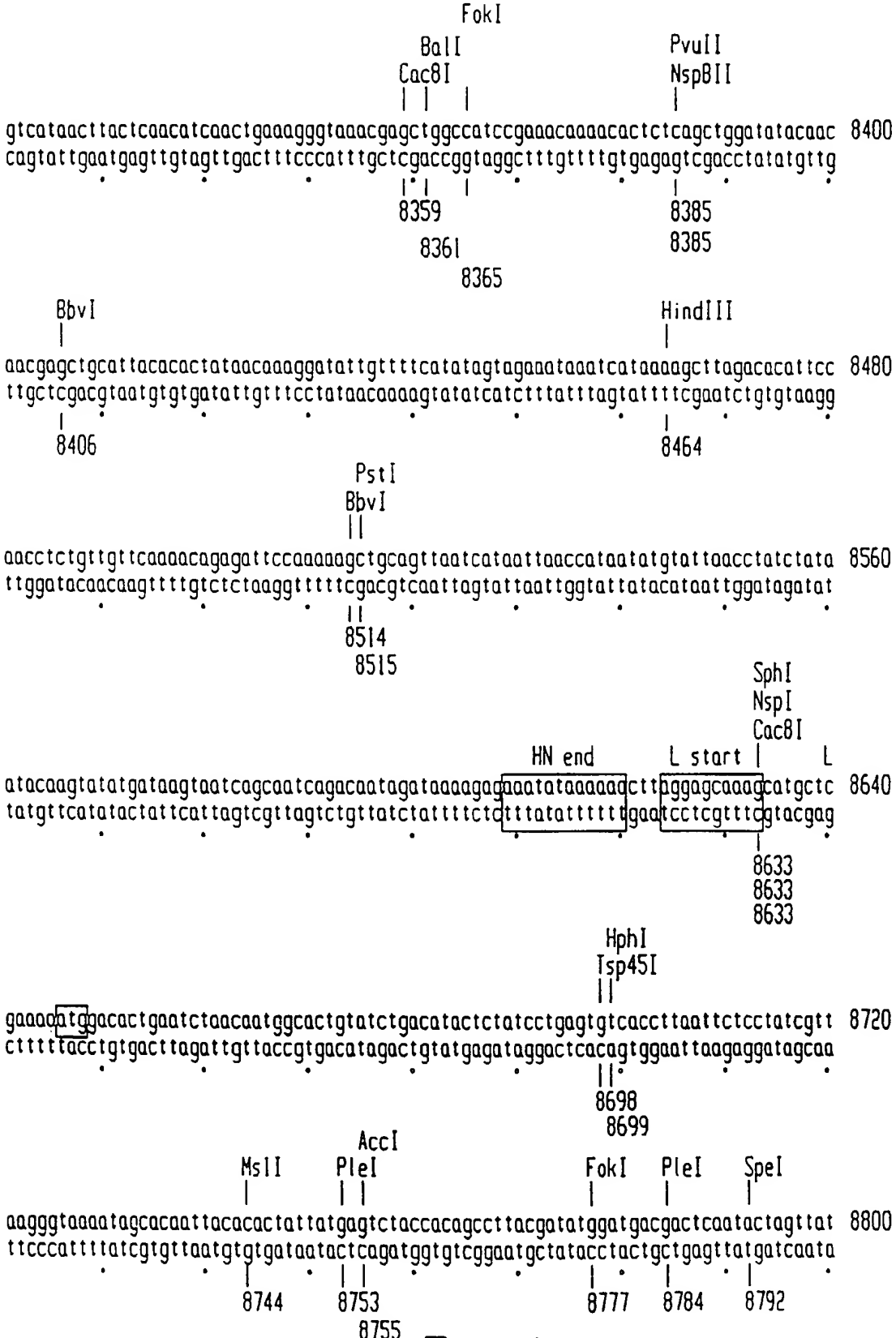
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Fig. 1q

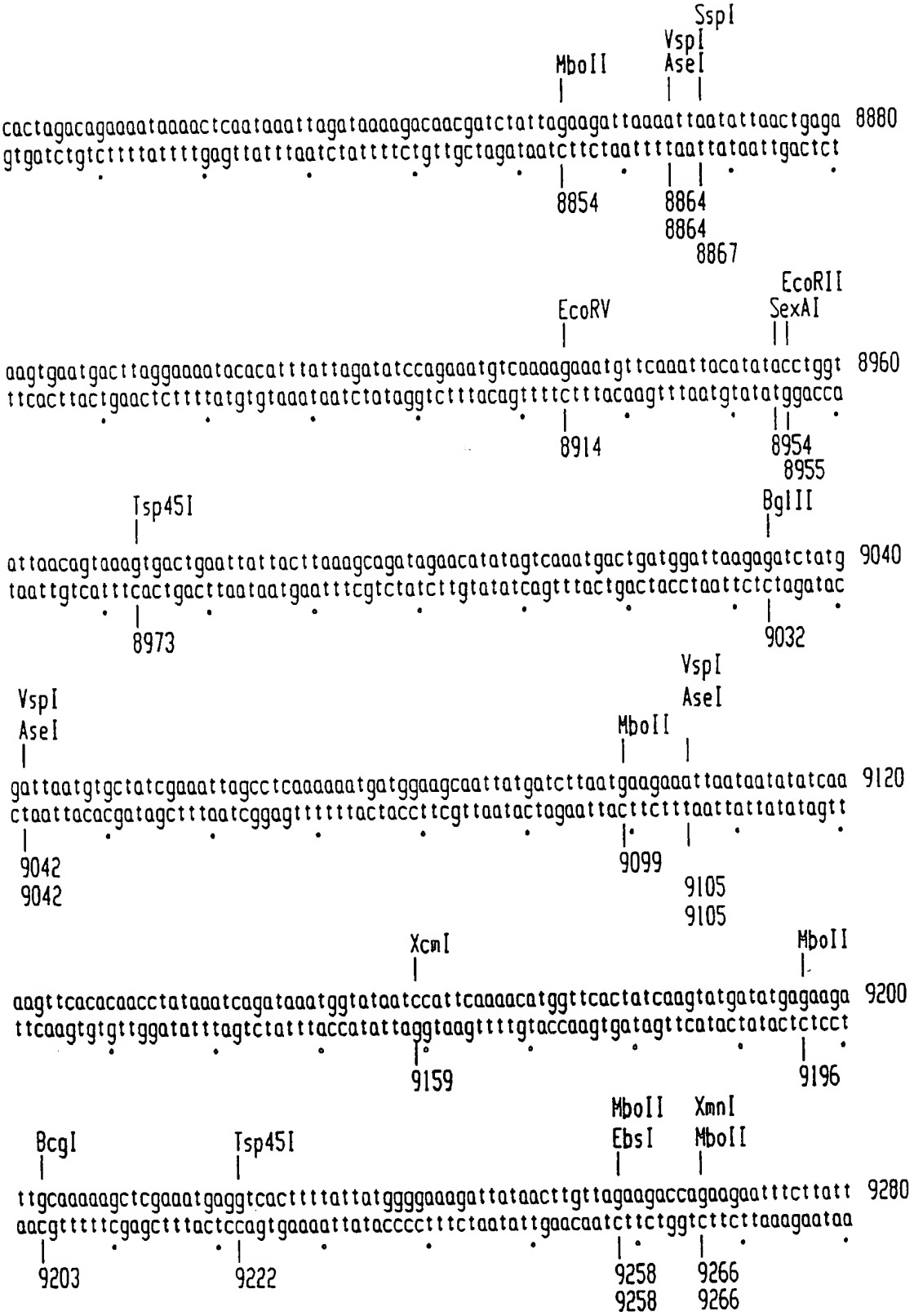
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Fig. 1r

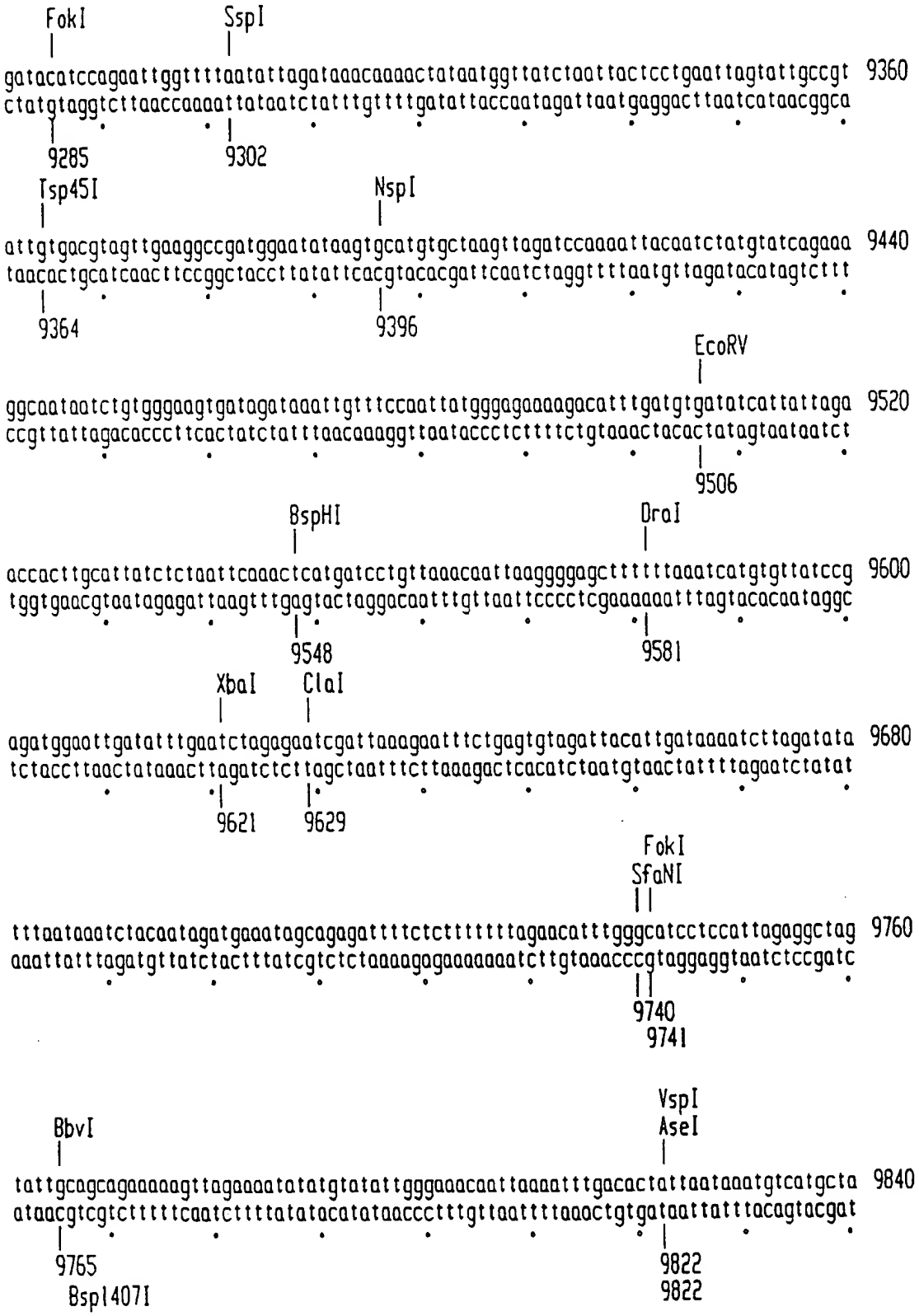
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Fig. 1s

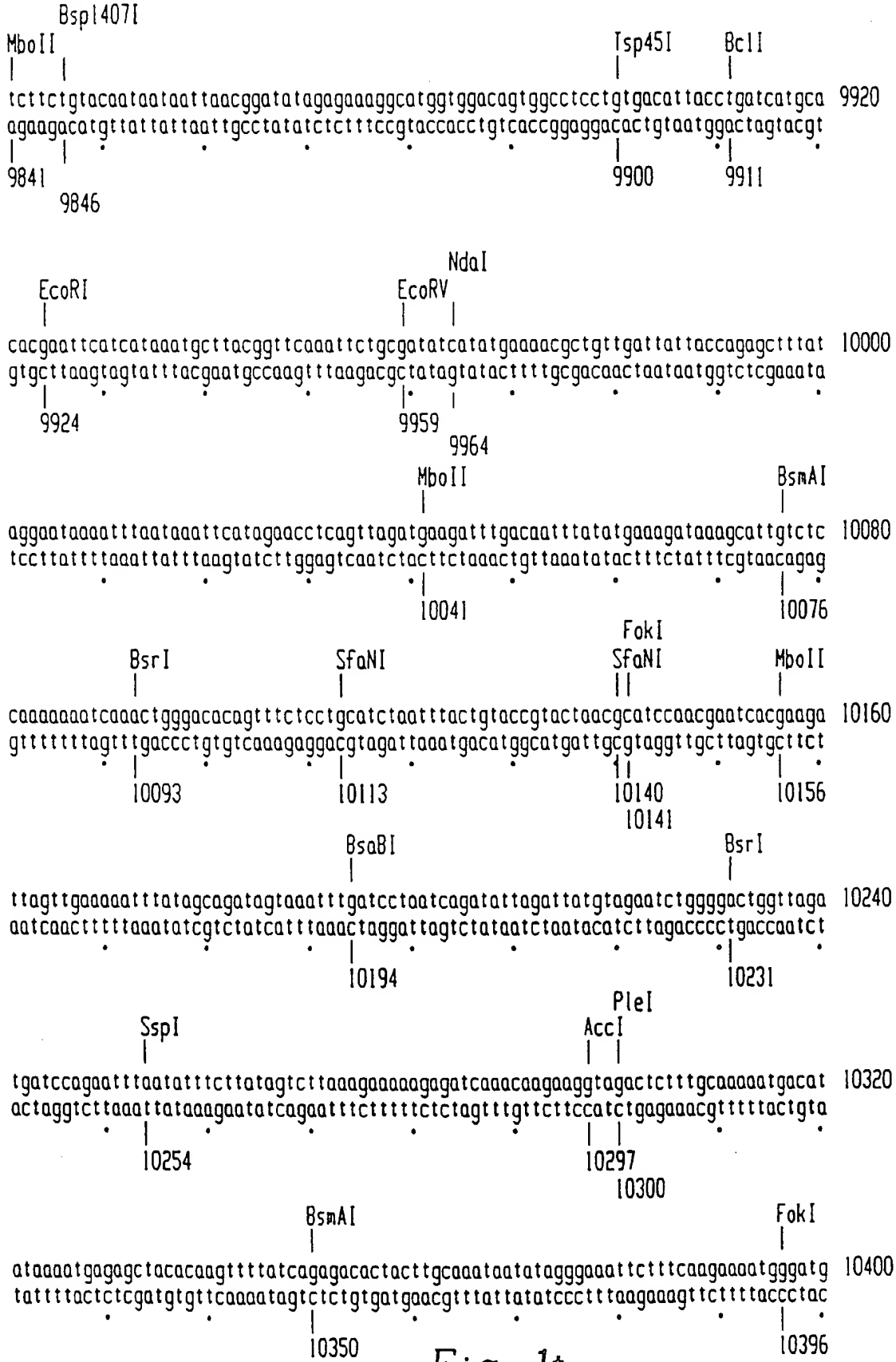
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Fig. 1t

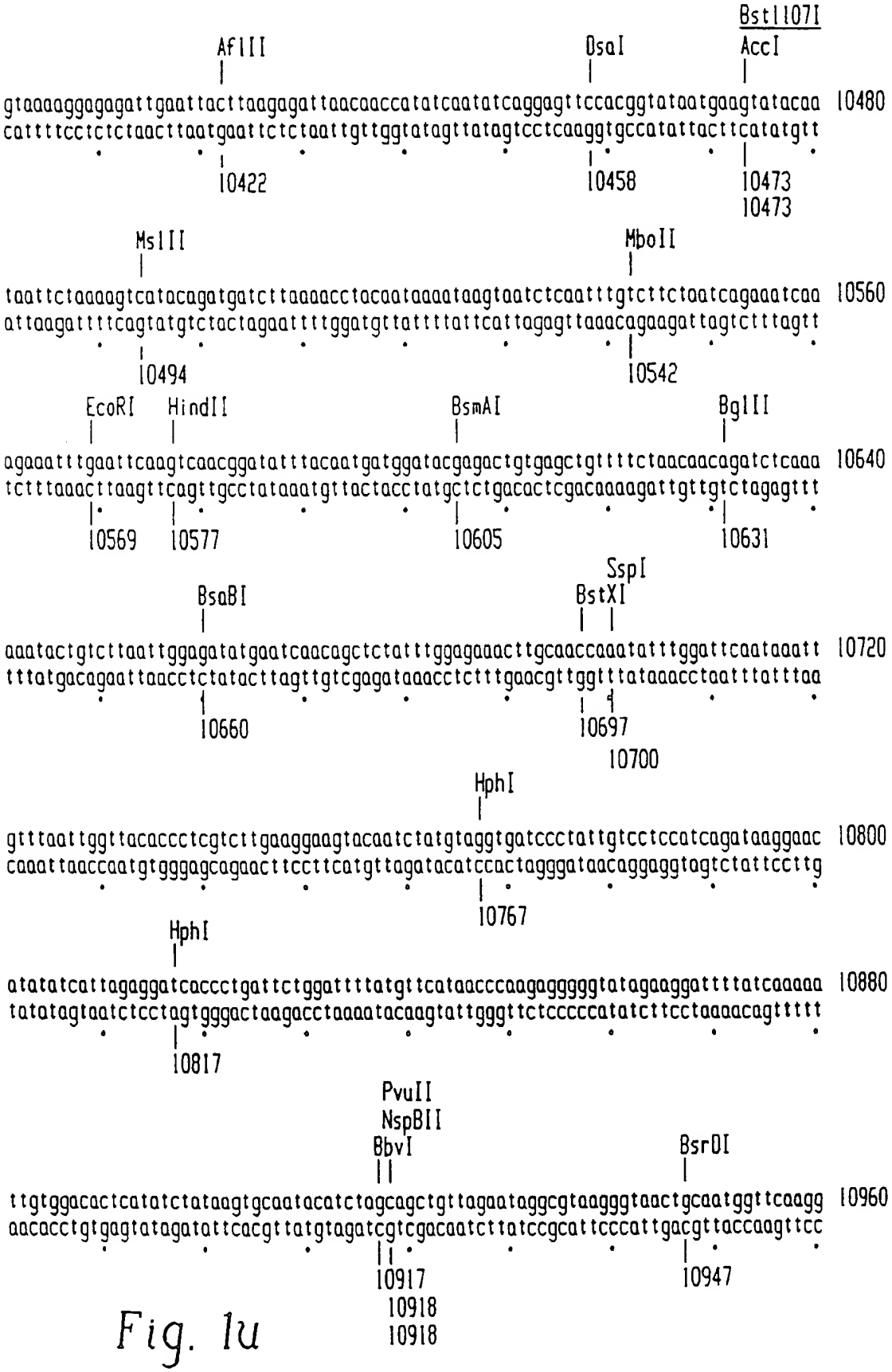
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Fig. 1u

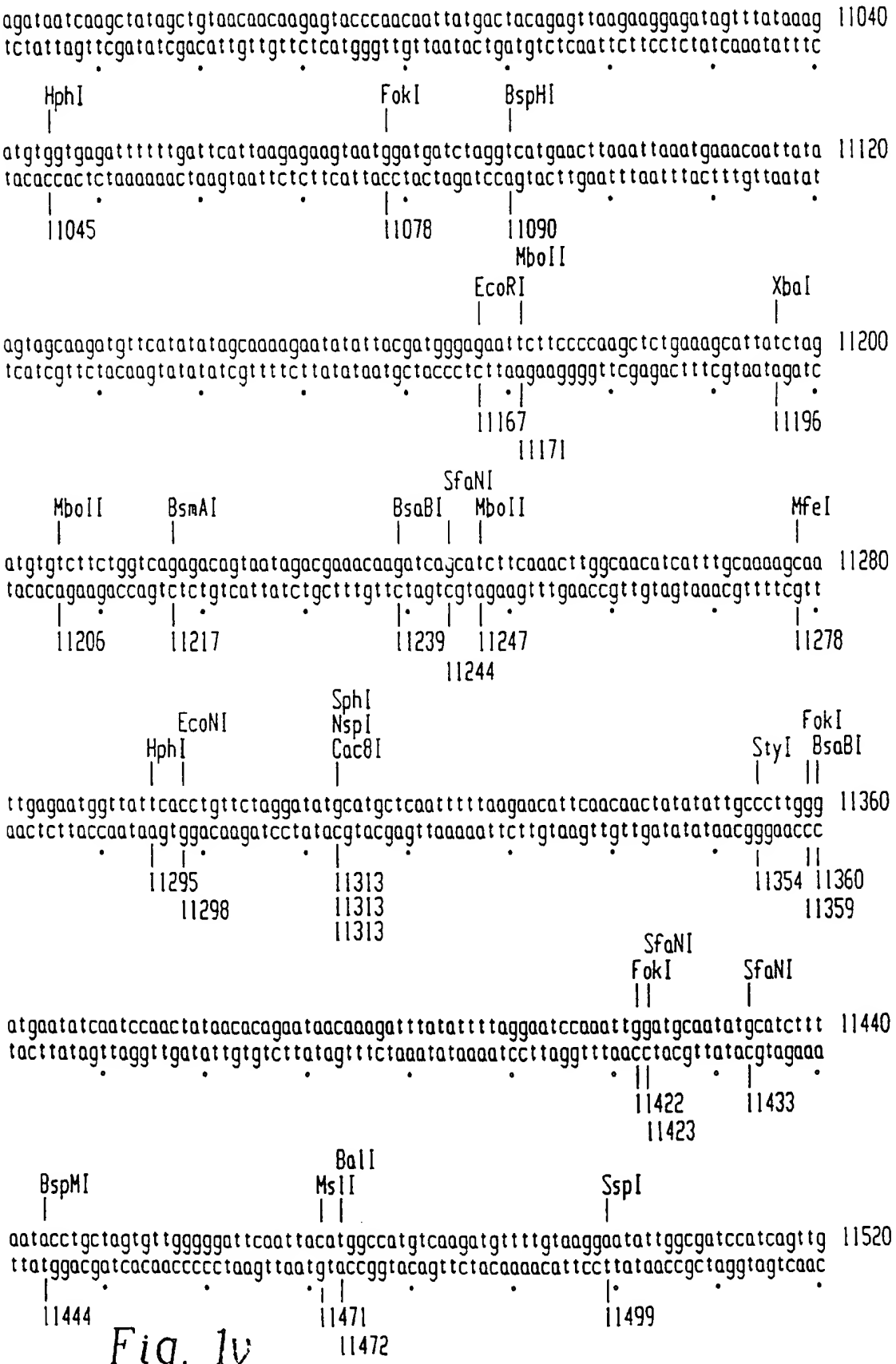
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Fig. 1v

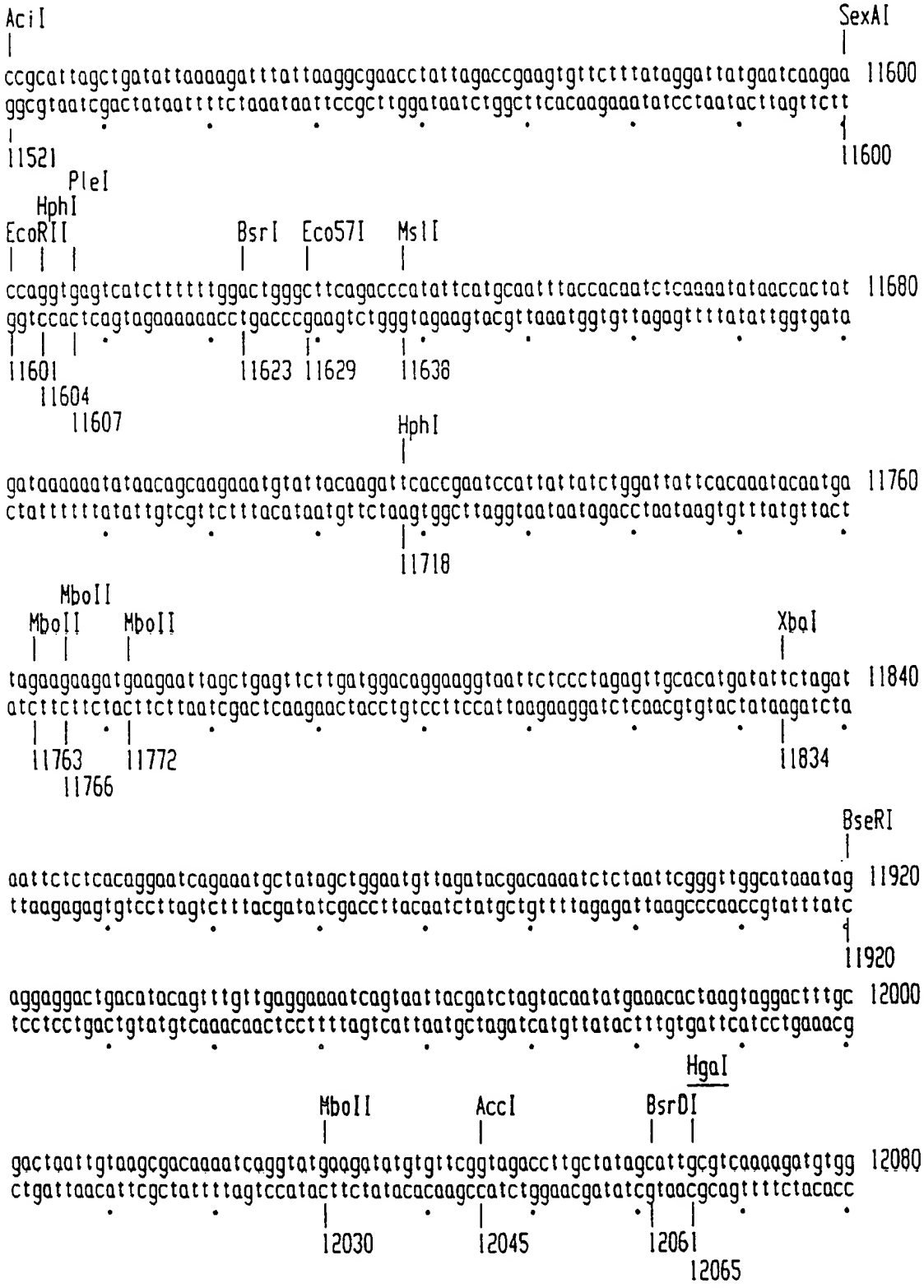
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Fig. 1w

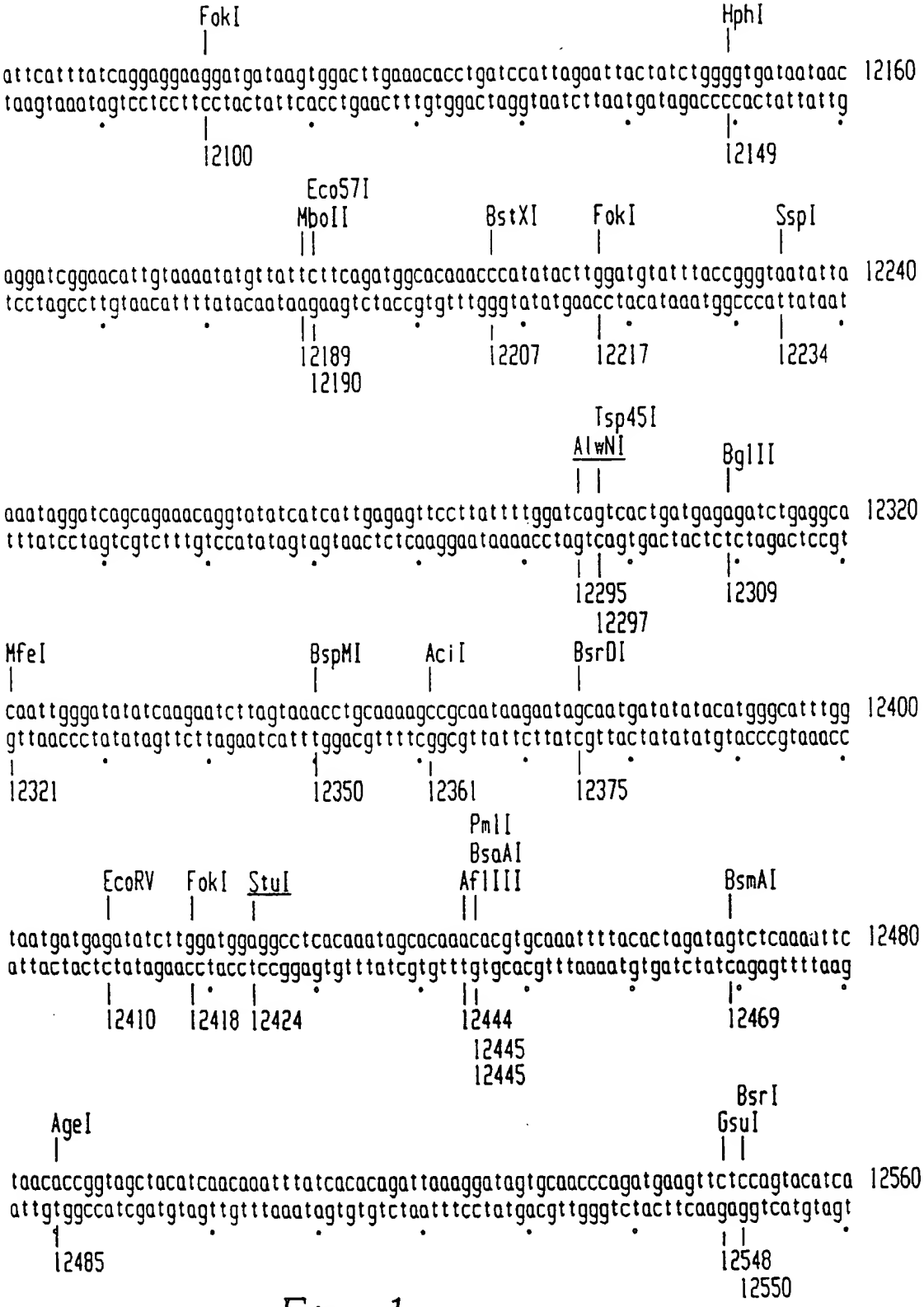
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Fig. 1x

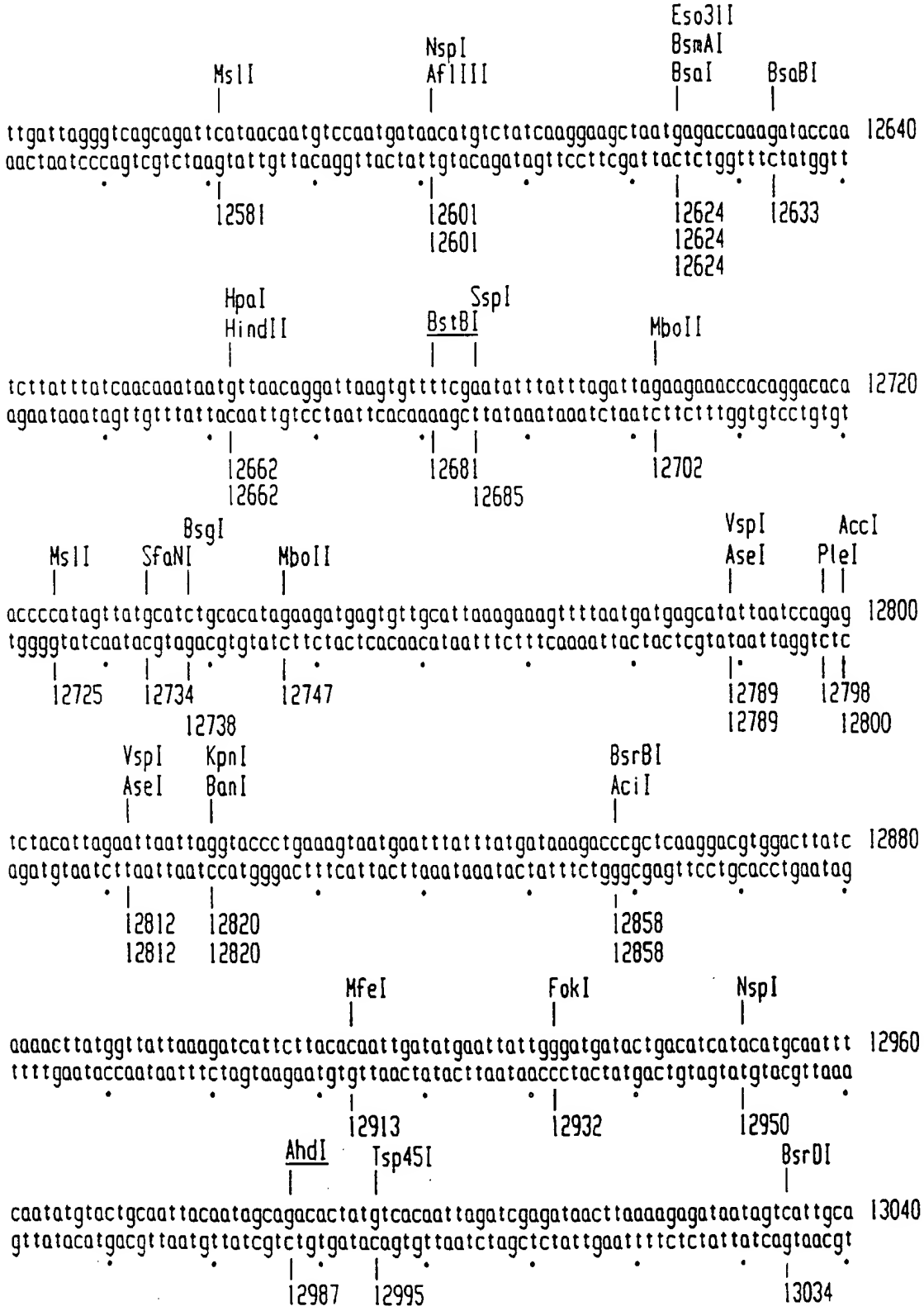
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Fig. 1y

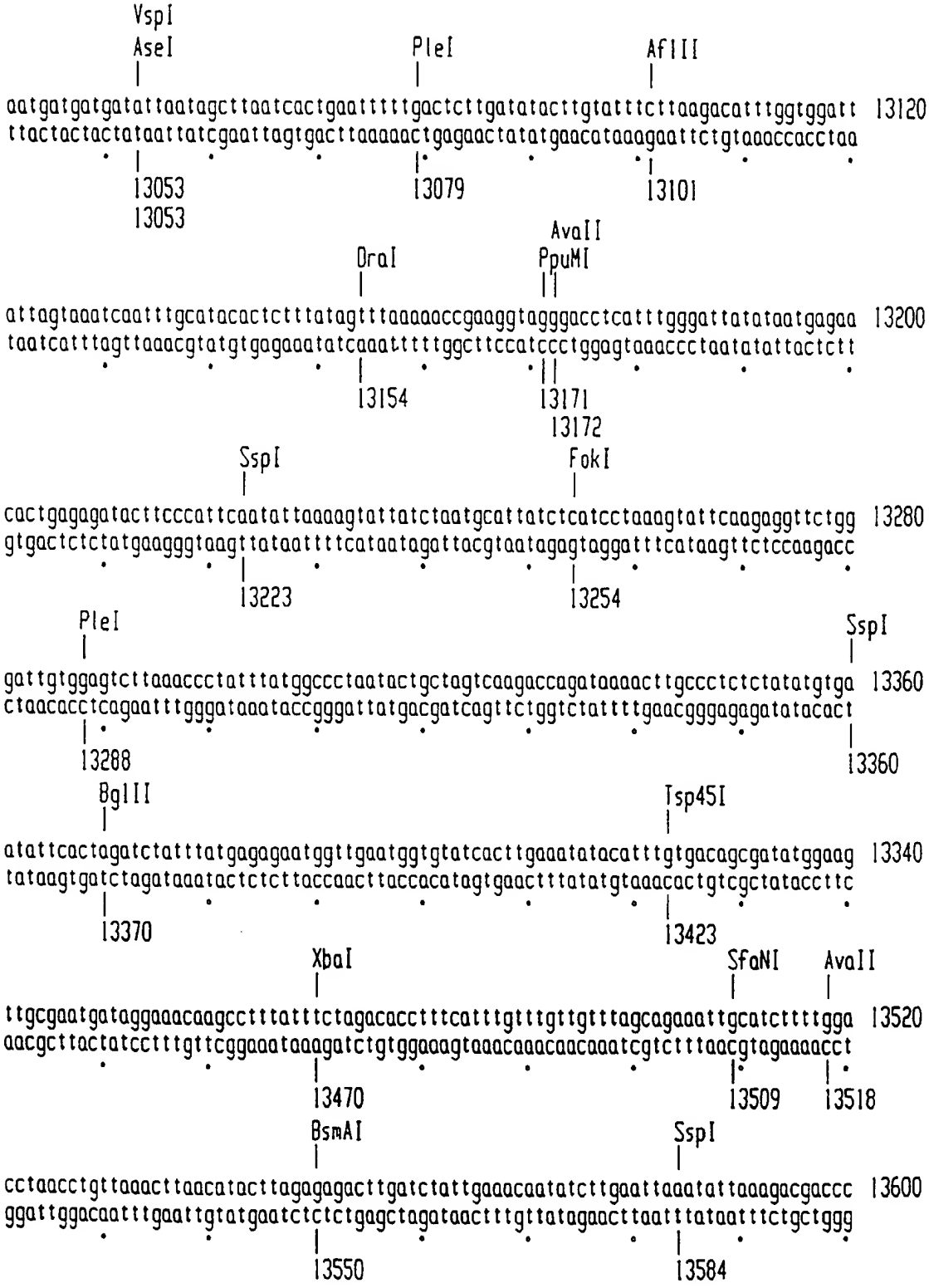
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Fig. 1z

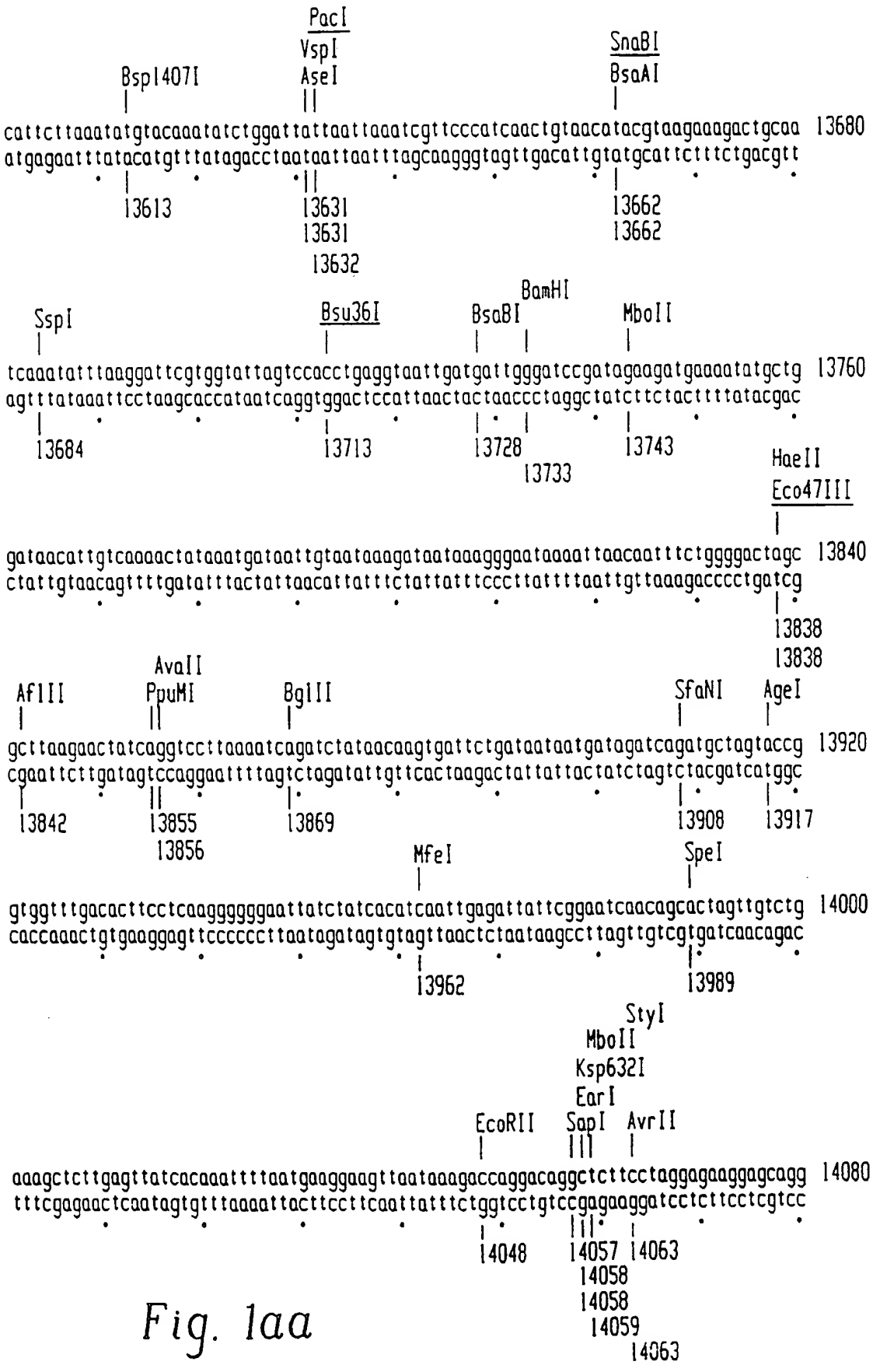
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Fig. 1aa

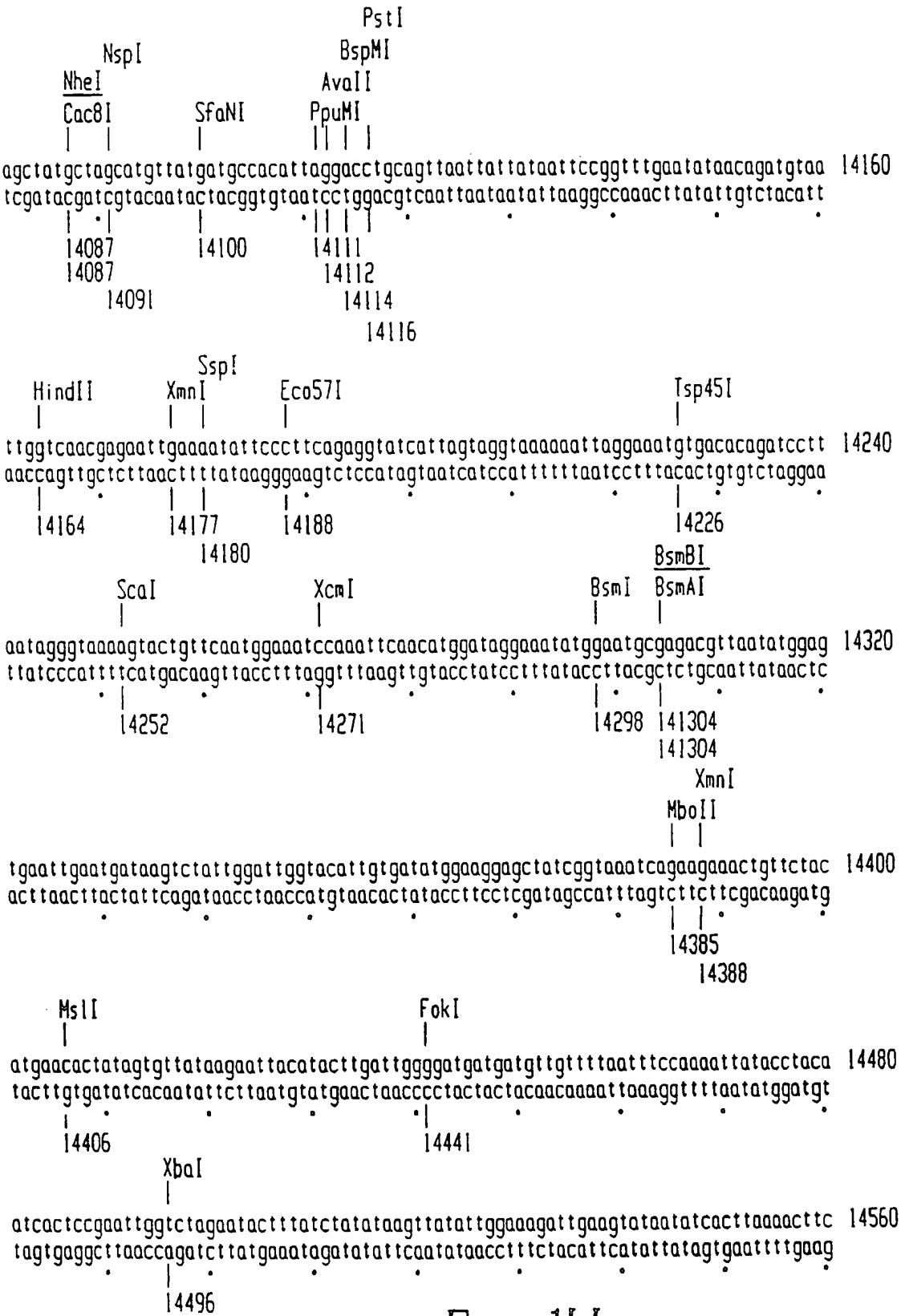
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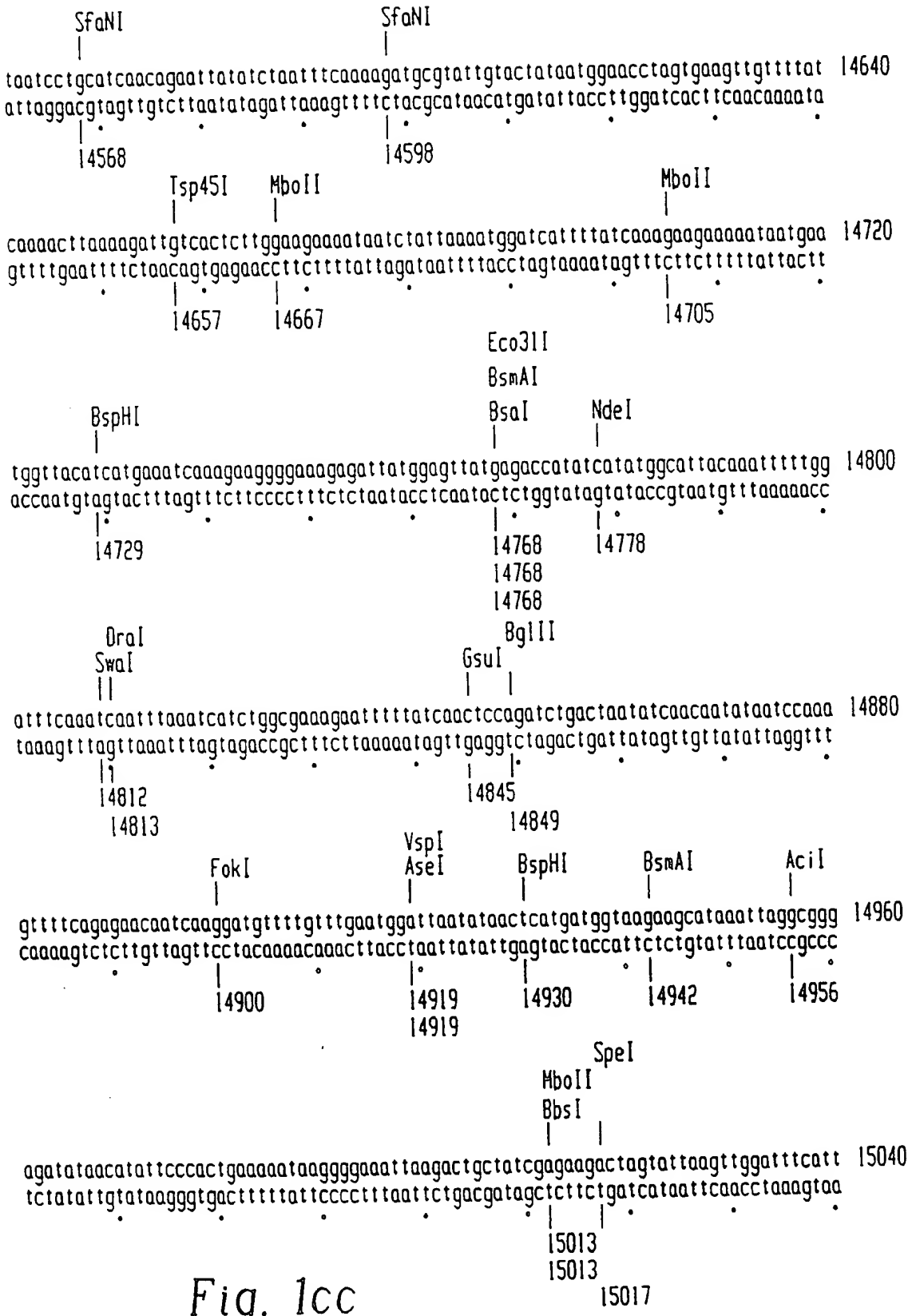
Fig. 16b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SwaI
BglII
GsuI
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14813
14845
14849
VspI
FokI
AseI
BspHI
BsmAI
AciI
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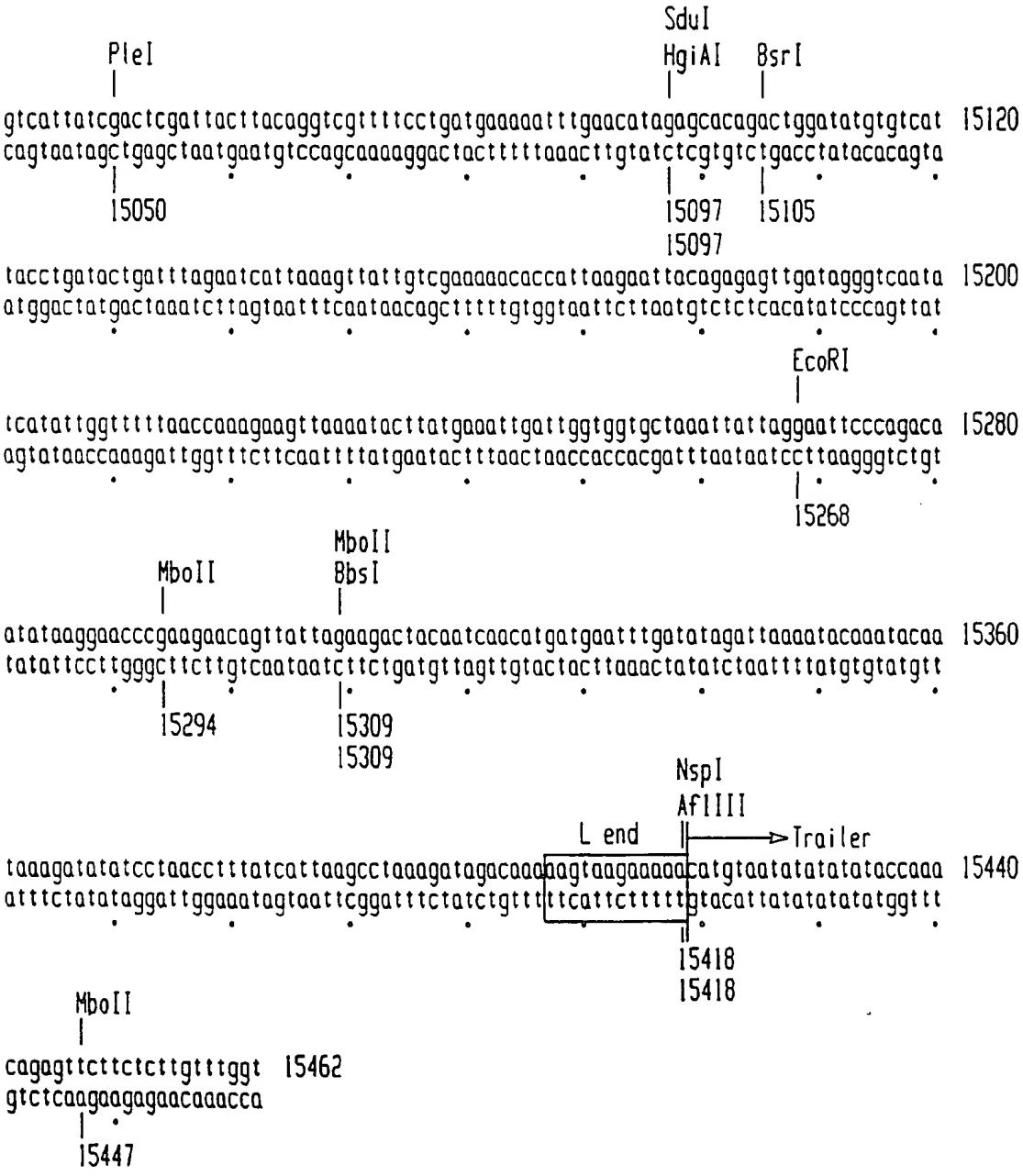
Fig. 1cc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15097
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MboII
BbsI
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15309
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AflIII
L end
Trailer
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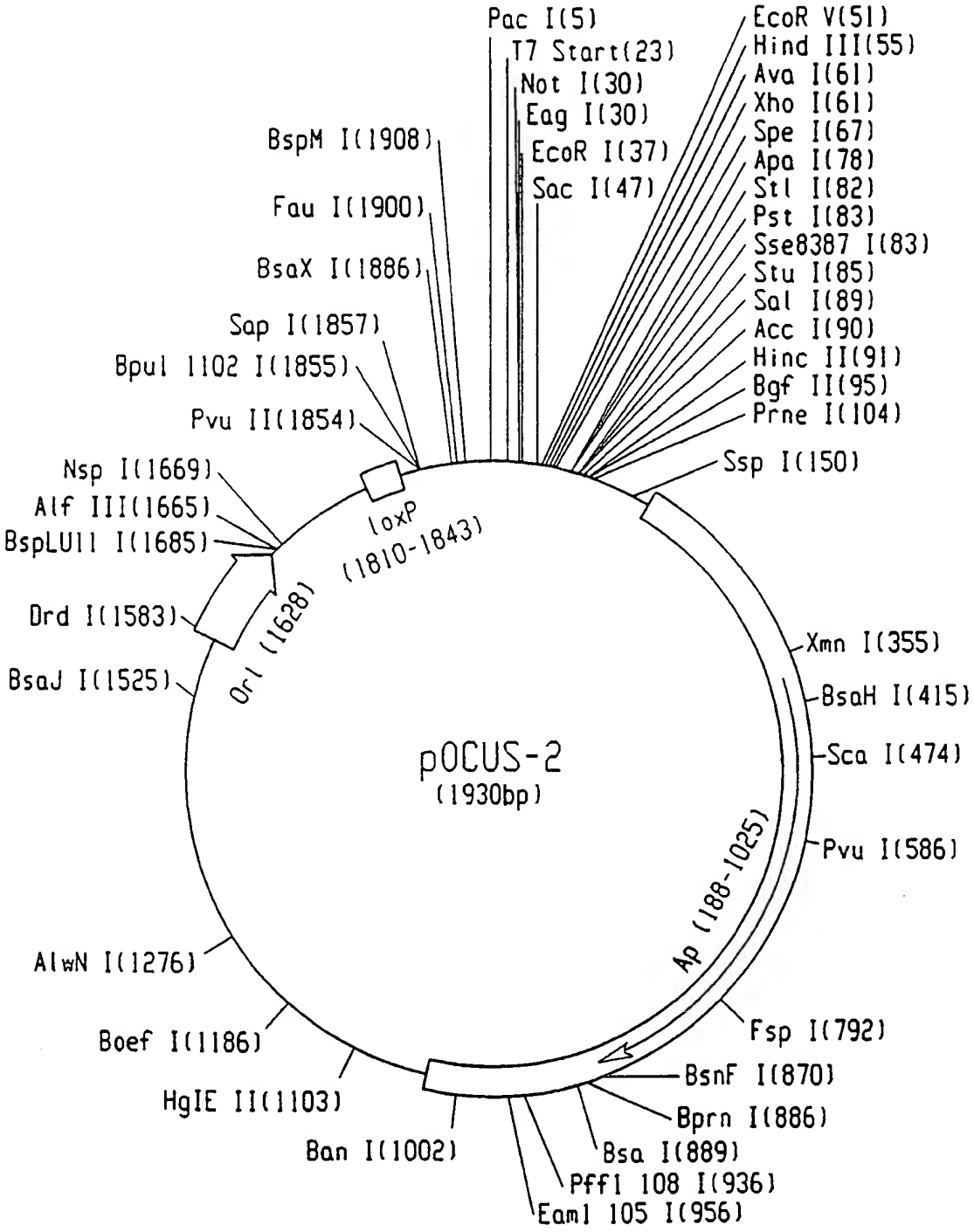
Fig. 1dd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Spe I(67)
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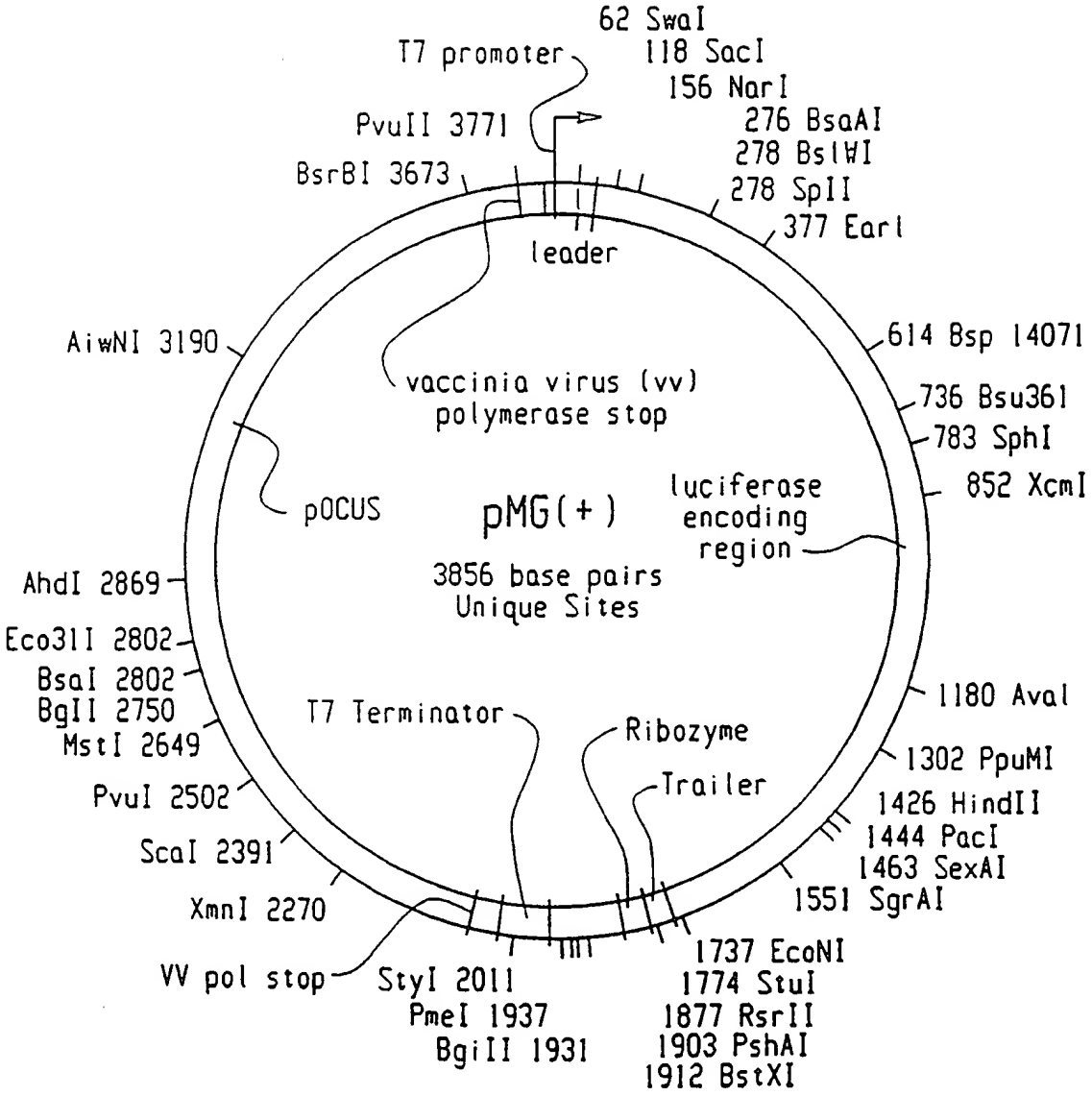
Fig. 2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1180 AvaI
1302 PpuMI
1426 HindII
1444 PacI
1463 SexAI
1551 SgrAI
1737 EcoNI
1774 StuI
1877 RsrII
1903 PshAI
1912 BstXI
Styl 2011
PmeI 1937
BgiII 1931
VV pol stop
XmnI 2270
ScaI 2391
PvuI 2502
MstI 2649
BglI 2750
BsaI 2802
Eco31I 2802
AhdI 2869
AiwNI 3190
BsrBI 3673
PvuII 3771
T7 promoter
leader
vaccinia virus (vv)
polymerase stop
pOCUS
pMG(+)
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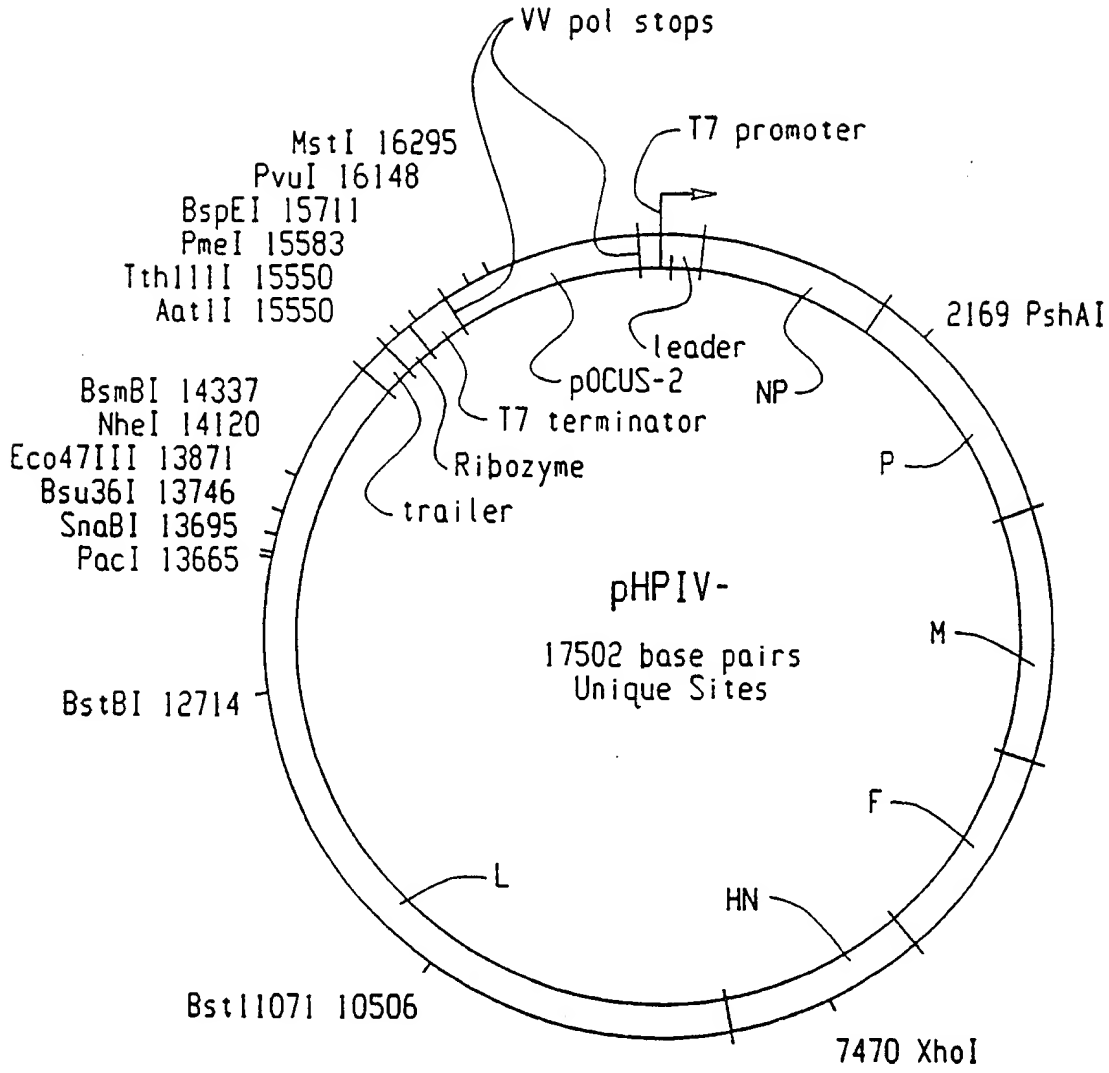
Unique Sites
luciferase
encoding
region
T7 Terminator
Ribozyme
Trailer

Fig. 3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PvuI 16148
BspEI 15711
PmeI 15583
Tth111I 15550
AatII 15550
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leader
pOCUS-2
NP
T7 terminator
BsmBI 14337
NheI 14120
Eco47III 13871
Bsu36I 13746
SnaBI 13695
PacI 13665
Ribozyme
trailer
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pHPIV-
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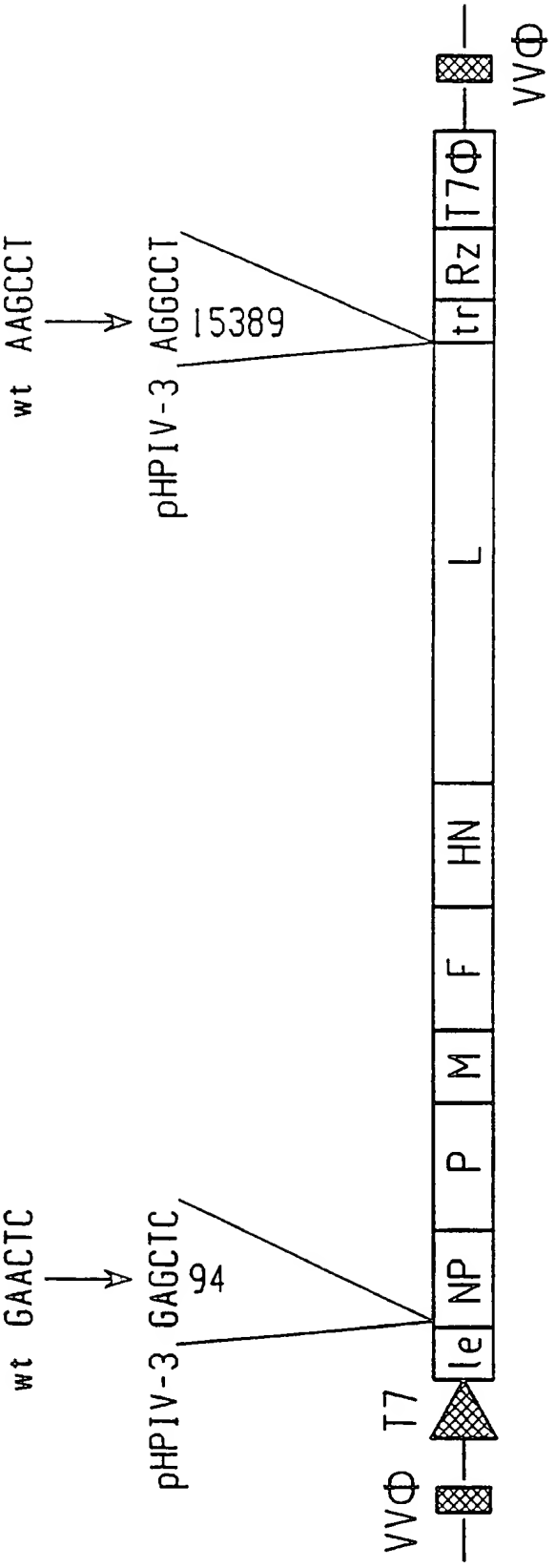
Unique Sites
M
BstBI 12714
F
L
HN
Bst11071 10506
7470 XhoI

Fig. 4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tr
Rz
L
HN
F
M
P
NP
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T7
VVΦ
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15389
wt GAACTC
pHPIV-3 GAGCTC
94

Fig. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/09270

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C07H 21/04; C12P 19/34; C12N 5/06, 5/08
US CL :536/23.72, 24.1; 435/91.1, 91.51, 325, 367
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 536/23.72, 24.1; 435/91.1, 91.51, 325, 367

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GARCIN et al. A Highly Recombinogenic System for the Recovery of Infectious Sendai Paramyxovirus From cDNA: Generation of a Novel Copy-Back Nondefective Interfering Virus. 1995, Volume 14, No. 24, pages 6087-6094, see the entire document.	1-20
Y	KATO et al. Initiation of Sendai Virus Multiplication From Transfected cDNA or RNA with Negative or Positive Sense. Genes to Cells. June 1996, Volume 1, pages 569-579, see the entire document.	1-20
Y	EP 0,702,085 A1 (AKZO NOBEL N.V.) 20 March 1996 (20/03/96), see the entire document and especially page 2, column 1, line 1, through page 4, column 6, line 34.	1-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 27 JULY 1998	Date of mailing of the international search report 01 SEP 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer BRENDA BRUMBACK Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)☆

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/09270

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/09270

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

DIALOG: Medline, BIOTECH, Conf. Papers, Euro, Japio, WPI, APS
search terms: genome, antigenome, RNA, DNA, cDNA, polymerase, promoter, ribozyme, T7, plasmid, parainfluenza, HPIV, paramyxovirus, L protein, P protein, NP protein, transfect?, mutat?

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-12, drawn to a recombinant HPIV clone and a method of using.

Group II, claims 13-17, drawn to a host cell for producing a recombinant HPIV clone.

Group III, claims 18-20, drawn to a method of introducing a site-specific mutation into the genome of HPIV.

The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The inventions are separate products, with different technical features. Rule 13.2 does not provide for multiple products.